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Stress tolerance

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088-Cryo-PROV

Stress Tolerance

Field of the invention

The present invention concerns a method for identifying and obtaining nucleic acids capable of modifying stress tolerance, particularly cold tolerance, in plants. The invention also concerns isolated nucleic acids so obtained. The invention further concerns a method for obtaining plants having modified stress tolerance and to plants obtained by the methods according to the invention. The invention also relates to a yeast strain having modified tolerance to cold stress.

Background

Environmental stress conditions, such as shortage or excess of solar energy, water or nutrients, high salinity and pollution (e.g., heavy–metal pollution), can have a major impact on plant growth and can significantly reduce plant yield. Osmotic stress, a type of environmental stress, may be induced by conditions of excess salinity, drought, excessive heat, cold or freezing.

Cold stress may be induced by temperatures below the range which allow optimal growth for a particular plant species. Each plant species or variety has an optimal growth temperature at which the growth rate is maximal; the further the deviation from this optimal growth temperature, the greater the stress on the plants. Many plant species, especially from tropical or subtropical regions, are sensitive to cold. For example, it has been estimated that the worldwide rice production would decrease by 40% if the worldwide mean temperature dropped only between 0.5 to 1.0°C (Salisbury and Ross, Plant Physiology. 4th ed. Wadsworth Publishing Company, Belmont, CA, 1992). Plants from temperate regions however have the ability to adapt their metabolism and to survive freezing temperatures after undergoing a process of adaptation to low but non-freezing temperatures, a process called cold acclimation. For instance non-acclimated rye typically does not survive temperatures below –5°C, but after cold acclimation it can withstand temperatures as low as –30°C. The process of cold acclimation involves altered expression of many genes. Plants may differ in their ability to withstand cold, which could lead to periodic but significant losses in plant productivity. As a consequence, the areas in which crops or horticultural plants can be cultivated is determined by assessing the risk of lower temperatures, relative to typical growth temperatures for any given plant.

The most prominent changes during cold acclimation include a reduction or cessation of growth, reduction of tissue water content (Levitt; Responses of Plants to Environmental Stresses, Vol. 1. 2nd edn. Academic Press. New York, NY 1980), transient increase in abscisic

acid (ABA) levels (Chen *et al.*, Plant Physiology **71**, 362 – 365, 1983), changes in membrane lipid composition (Lynch and Steponkus, Plant Physiology **83**, 761 – 767, 1987; Uemura and Steponkus, Plant Physiology **104**, 479 – 496, 1994), the accumulation of compatible osmolytes such as proline, betaine, polyols and soluble sugars, and increased levels of antioxidants (Koster and Lynch, Plant Physiology **98**, 108 – 113, 1992; Kishitani *et al.*, Plant, Cell and Environment **17**, 89 – 95, 1994; Murelli *et al.*, Physiologia Plantarum **94**, 87 – 93 1995; Nomura *et al.*, *Euphytica* **83**, 247 - 250, 1995; Dörffling *et al.*, Plant Molecular Biology **23**, 221 – 225, 1997; Tao *et al.*, Cryobiology **37**, 38 - 45, 1998).

Various methods for the identification and isolation of genes or proteins differentially expressed during cold stress are known. For example, mapping techniques allow determination of chromosome locations of genes involved in cold tolerance (Pan *et al.*, Theoretical and Applied Genetics **89**, 900 - 910, 1994; Galiba *et al.*, Theoretical and Applied Genetics **90**, 1174 – 1179, 1995). Another approach involves mutational analysis in which mutants that have an altered response to cold tolerance are isolated and characterized. For example, *eskimo1*, conferring improved freezing tolerance of 2°C over acclimated wild-type plants, was isolated from a collection of 800000 Ethyl Methyl Sulphonate (EMS)- mutagenised *Arabidopsis* lines that were screened for constitutively freezing-tolerant mutants (Xin and Browse, PNAS **95**, 7799 – 7804, 1998). Conversely, plant lines were screened for mutants defective in cold acclimation (Warren *et al.*, Plant Physiology **111**, 1011 – 1019, 1996; Knight *et al.*, Plant Cell **8**, 489 – 503, 1996). cos-, los- and hos-mutants (for respectively constitutive, low and high expression of osmotically responsive genes) were isolated using a combination of mutagenesis and reporter gene activation (Ishitani *et al.*, Plant Cell **9**, 1935 - 1949, 1997; Ishitani *et al.*, Plant Cell **10**, 1151 – 1161, 1998; Lee *et al.*, Plant Journal **17**, 301 – 308, 1999). One of the drawbacks of mapping and the mutant analysis strategy is that they do not directly result in the isolation of nucleic acids coding for cold-induced genes. Another strategy, using differential screening of cDNA libraries and related techniques, has in the past yielded several cold induced genes from different plant species (reviewed in Xin and Browse, Plant, Cell and environment **23**, 893 – 902, 2000). Many of those genes have known functions and can be grouped as being involved in drought stress, in signal transduction pathways, or as being related to heat shock proteins, molecular chaperones, "antifreeze proteins" or regulatory proteins. Several of the genes are highly expressed during cold stress and are commonly referred to as *COR* (COld Regulated) genes (Tomashow, Annual Review of Plant Physiology and Plant Molecular Biology **50**, 571 - 599, 1999).

Strategies used to engineer cold resistant plants include accumulation of osmoprotectants such as mannitol (US 6,416,985), proline (US 6,239,332), trehalose (US 6,323,001) or glycine-

betaine (Hayashi *et al.*, Plant Journal **12**, 133 – 142, 1997; US 6,281,411). Other approaches involve manipulating the signal transduction pathway controlling the stress response (WO 01/77355), including use of transcription factors (WO 01/77311, US 6,417,428, WO 02/44389, US 5,891,859). Furthermore a number of genes have been used to enhance cold resistance. Examples are members of the COR group (*COR15a*: US 5,296,462, US 5,356,816), a cell cycle related gene (WO 01 /77354), protein kinase related proteins (WO 01/77356), the LEA-like protein CAP85 (US 5,837,545) and use of a phospholipid binding protein (WO 02/00697).

Signal transduction pathways leading to cold acclimation and the identity of the genes that confer resistance to cold stress in plants remain largely unknown. A recent study in yeast by de Jesus Ferreira et al. (2001), in which transposon mutagenesis was employed followed by a cultivation of the mutants at 15°C, identified 10 different genes responsive to cold tolerance. The identified genes include a gene coding for a glutamate synthase (YDL171C), a GTP binding protein (*YML121W*), a GSK-3 Ser/Thr protein kinase (*YNL307C*) and a component of TFIID (*YLR399C*). Three of the genes were previously described as cold responsive (*YLR399C, YML121W, YNL307C*) and four of the isolated genes were also involved in resistance to salt stress.

Yeast has been used for screening plant genes that confer resistance to salt stress. For example, a salt-sensitive yeast strain (JM26) has previously been transformed with a cDNA library from salt-stressed sugar beet and used to screen for clones having increased salt tolerance (WO 02/52012). The transformed yeast cells were grown on a rich medium (YPD) or on a synthetic medium plus methionine and leucine (SD), supplemented with 0.15 M NaCl or with 20 mM LiCl. Putative positive clones showing better growth on the selective media compared to the non-transformed yeast strain were isolated and further characterised.

Summary of the invention

The present invention provides a method for screening for nucleic acids involved in stress responses in a plant, which method involves screening in yeast for plant genes involved in modifying tolerance/resistance to temperature stress. The present invention also provides new plant genes identified by this screen and polypeptides encoded by these genes. Also provided are methods for producing plants having modified tolerance or resistance to environmental stress conditions, comprising introduction of the above-mentioned genes into plants. Also provided are plants having modified tolerance or resistance to environmental stress conditions, which plants are transformed with the gene according to the invention.

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Detailed description of the invention

According to a first embodiment of the present invention, there is provided a screening method for identifying nucleic acids capable of modifying tolerance or resistance to stress conditions in plants, which method comprises the steps of:

(i) providing a cDNA library of coding sequences from salt-treated plants;
(ii) introducing these coding sequences in an expressible format into yeast cells;
(iii) growing the yeast cells under conditions of stress other than salt stress;
(iv) identifying differences between transgenic yeast cells and wild type yeast cells, preferably identifying differences in growth rate;
(v) isolating nucleic acids from the transgenic yeast cells that differ from the wild type yeast cells.

The present invention demonstrates that yeast cells transformed with cDNA from salt stressed plants can be used to isolate genes capable of conferring tolerance against cold stress in plants.

The terms "tolerance" and "resistance" are used interchangeably herein.

The first step of the screening method involves providing a cDNA library of coding sequences from salt-treated plants. According to a preferred feature of the present invention, the cDNA library is made from a salt treated halophytic plant or a part thereof, preferably from a salt treated sugar beet plant or a part thereof, more preferably from leaves of salt treated *Beta vulgaris* plants. Sugar beet (*Beta vulgaris*), a relatively halophytic crop plant, provides a potentially good source of cold tolerance genes. Although the present invention is exemplified by use of a sugar beet cDNA library, it is to be understood that other halophytic plants could equally serve the same purpose. The preparation of cDNA libraries is a routine technique well known in the art. The cDNA library preferably comprises copies of essentially all mRNA of the plant cell. Advantageously, coding sequences alone are sufficient.

The second step of the screening method involves introducing the coding sequences into yeast cells. Methods for transformation of yeast, such as electroporation or treatment with Lithium Acetate, and for expressing genes in yeast, including yeast vectors, such as pYES, are well known in the art (see e.g. *Current Protocols in Molecular Biology*, Unit 13 (Ausubel *et al.*, 1994) and the *Guide to Yeast Genetics and Molecular Biology* (Guthrie and Fink, 1991)). Advantageously, coding sequences may be introduced and expressed in yeast using any of several known methods, with the aim of testing tolerance or resistance to stress conditions. According to a preferred feature of the present invention, a vector based on the λ phage is

employed, more preferably λPG15 is used for introducing and expressing coding sequences in yeast. Phage λPG15 comprises the excisable expression plasmid pYPGE15 which may be used directly for both *Escherichia coli* and yeast complementation (Brunelli and Pall, Yeast **9**, 1309 – 1318, 1993). A plasmid cDNA library can be recovered from λPG15 using the *cre-lox* recombinase system (Brunelli and Pall, Yeast **9**, 1309 – 1318, 1993). Preferably, the yeast cells are *Saccharomyces cerevisiae*, more preferably the diploid wild type strain W303 and its diploid mutant deficient for glycerol phosphate dehydrogenase (*gpd1*). The yeast strain W303 has the genotype MATa/MATα, ADE2/ade2, CAN1/can1-100, CYH2/cyh2, his3-11,15/his3-11,15, LEU1/leu1-c, LEU2/leu2-3,112, trp1-1:URA3:trp1-3'D /trp1-1, ura3-1/ura3-1, and originates from the parent strains W303-1A and W303-1B (Primig et al., *Nat. Genet.* **26**, 415 – 423, 2000). The W303 gpd1 mutant was unexpectedly more cold tolerant than the W303 wild type strain (see Figure 1). For this reason the wild type strain was used in the screening, while the *gpd1* mutant strain served as a standard for comparison. It was thus expected that nucleic acids conferring cold tolerance would enhance the growth of the wild type yeast cells to a comparable or better level to that of the gpd1 mutant.

Advantageously, the *gpd1* gene can be used for enhancing cold tolerance of yeast, for example baker's yeast. Yeast is known to be sensitive to cold stress. Freezing stress in particular has a negative impact on the quality of yeast as a leaven. Yeast cells that have been mutated or engineered such that the glycerol phosphate dehydrogenase (*gpd1*) gene is inactivated (using techniques known in the art) are surprisingly more tolerant to cold and/or freezing stress than wild type yeast. This trait can be of benefit in, for example, the baking or brewing industries. The present invention thus also provides a method for increasing cold tolerance of yeast cells, comprising modulating expression in yeast of a nucleic acid encoding a glycerol phosphate dehydrogenase and/or modulating activity of a glycerol phosphate dehydrogenase. Preferably, the expression of glycerol phosphate dehydrogenase is downregulated or inhibited. The invention furthermore provides for the use of a *gpd1* gene for altering the stress tolerance of yeast. The stress-tolerant yeast cells can be used in purified form (for example leaven) or in compositions (for example dough).

The third step of the screen involves growing the yeast cells under stressed conditions. Yeast cells transformed with cDNA of the salt stressed sugar beet were plated onto a suitable medium and grown under cold stress. After a certain period of time colonies that were able to grow under these conditions of cold were selected and their cold tolerance was retested by growing the transgenic cells again under cold stress conditions. Advantageously, the cDNA from salt treated plants may also be a suitable basis for finding genes capable for conferring tolerance against other stresses. This may be achieved simply by growing the yeast cells in

step (iii) above in conditions of stress determined by the type of gene sought. For example, in order to identify genes conferring tolerance or resistance to heat stress, the yeast cells would be grown in conditions of heat. According to a preferred feature of the invention, the stress is preferably cold stress, but similar results may also be obtained when using other types of stress, such as drought stress, osmotic stress, oxidative stress or temperature stress other than cold stress (such as heat or freezing stress), with the proviso that the stress is not salt stress. It was then determined whether the stress tolerance originated from the transgene and not from a mutation in the host genome. To this end, the plasmid comprising the transgene was cured from a transgenic cold tolerant yeast clone and it was verified whether the cold tolerance had disappeared too; secondly the plasmid comprising the transgene was isolated from a transgenic cold tolerant yeast clone and reintroduced into a non-transgenic yeast strain, whereafter the cold tolerance of the newly transformed yeast strain was compared to the non-transformed yeast strain.

The fourth step in the screening method is the identification of fast growing yeast cells. Yeast cells transformed with a plant nucleic acid conferring stress resistance were identified based on their ability to grow faster under stress conditions than yeast cells not transformed with such a nucleic acid, although other selection criteria may also be used, depending on the type of stress that is applied.

Finally, in the last step of the screening method nucleic acids conferring stress tolerance are isolated from the yeast host and characterised. Methods for isolating nucleic acids from yeast and sequencing these nucleic acids are known to those skilled in the art.

The nucleic acids obtained by the screening method of the invention are herein referred to as CRYO genes, encoding CRYO proteins.

Another embodiment of the present invention provides nucleic acids obtainable by the screening method according to the present invention, which nucleic acids can be used to modify stress tolerance or resistance in plants and/or yeast.

The screening method according to the invention identified several nucleic acids hitherto unknown. The present invention therefore also provides an isolated nucleic acid selected from:

(i) a nucleic acid encoding a protein as given in any one of SEQ ID NO 2, 4, 6, 8 and 10;

(ii) a nucleic acid as given in any one of SEQ ID NO 1, 3, 5, 7, 9 or the complementary strand thereof;

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(iii) nucleic acids which are allelic variants to the nucleic acids defined in any of (i) to (ii);

(iv) nucleic acids which are splice variants to the nucleic acids defined in defined in any one of (i) to (iii), and;

(v) nucleic acids which hybridise, preferably under stringent conditions, to polynucleotides defined in (i) to (iv);

(vi) a portion of a nucleic acid according to any of (i) to (v) above, which portion preferably encodes a protein having similar functional activity to the full length sequence.

The terms "nucleic acid(s)", "nucleotide sequence(s)", "gene(s)", "polynucleotide(s)" and "nucleic acid molecule(s)" are used herein interchangeably to refer to ribonucleotides or deoxyribonucleotides or a combination of both, in a polymeric form of any length. The terms also include double-stranded and single-stranded DNA and RNA. Also included are known nucleotide modifications such as methylation, cyclization and 'caps' and substitution of one or more naturally occurring nucleotides with an analogue such as inosine. The terms also encompass peptide nucleic acids (PNAs).

Advantageously, the nucleic acids according to the invention may be produced using recombinant or synthetic means, such as, for example, PCR cloning mechanisms. Generally, such techniques as defined herein are well known in the art, for example as described in Sambrook et al. (Molecular Cloning: a Laboratory Manual, 2001). Polynucleotides may also be synthesized by well-known techniques as described in the technical literature. See, e.g., Caruthers et al., Cold Spring Harbor Symp. Quant. Biol. **47** 411-418 (1982), and Adams et al., J. Am. Chem. Soc. **105** 661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

A nucleotide sequence encoding a protein (gene, coding sequence, open reading frame or ORF) is a nucleotide sequence that can be transcribed into mRNA and/or translated into a polypeptide when present in an expressible format, i.e. when the coding sequence or ORF is placed under the control of appropriate control sequences or regulatory sequences. A coding sequence or ORF is bounded by a 5' translation start codon and a 3' translation stop codon. A coding sequence or ORF can include, but is not limited to RNA, mRNA, cDNA, recombinant nucleotide sequences, synthetically manufactured nucleotide sequences or genomic DNA. The coding sequence or ORF can be interrupted by intervening nucleic acids.

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA and/or translated to produce a protein, either constitutively or following induction by an intracellular or extracellular signal, such as an environmental stimulus or stress (mitogens, anoxia, hypoxia, temperature, salt, light, dehydration, etc) or a chemical compound such as IPTG (isopropyl-β-D-thiogalactopyranoside), or such as an antibiotic (tetracycline, ampicillin, rifampicin, kanamycin), hormone (e.g. gibberellin, auxin, cytokinin, glucocorticoid, brassinosteroid, ethylene, abscisic acid etc), hormone analogue (indolacetic acid (IAA), 2,4-D, etc), metal (zinc, copper, iron, etc), or dexamethasone, amongst others. As will be known to those skilled in the art, expression of a functional protein may also require one or more post-translational modifications, such as glycosylation, phosphorylation, dephosphorylation, or one or more protein-protein interactions, amongst others. All such processes are included within the scope of the term "expressible format".

Genes and coding sequences essentially encoding the same protein but isolated from different sources can consist of substantially divergent nucleic acids. Reciprocally, substantially divergent nucleic acids can be designed to effect expression of essentially the same protein. These nucleic acids are the result of e.g. the existence of different alleles of a given gene, or of the degeneracy of the genetic code or of differences in codon usage. Differences in preferred codon usage are illustrated in http://www.kazusa.or.jp/codon. Allelic variants are further defined as to comprise single nucleotide polymorphisms (SNPs) as well as small insertion/deletion polymorphisms (INDELs, having a size of usually less than 100 bp). SNPs and INDELs form the largest set of sequence variants in naturally occurring polymorphic strains of most organisms. Additionally or alternatively, in particular conventional breeding programs, such as for example marker assisted breeding, it is sometimes practical to introduce allelic variation in the plants by mutagenic treatment of a plant. One suitable mutagenic method is EMS mutagenesis. Identification of allelic variants then takes place by, for example, PCR. This is followed by a selection step for selection of superior allelic variants of the sequence in question and which give rise to altered growth characteristics. Selection is typically carried out by monitoring growth performance of plants containing different allelic variants of the sequence in question (for example SEQ ID NO 1, 3, 5, 7 or 9). Monitoring growth performance can be done in a greenhouse or in the field. Further optional steps include crossing plants in which the superior allelic variant was identified with another plant. This could be used, for example, to make a combination of interesting phenotypic features. According to another aspect of the present invention, advantage may be taken of the nucleotide sequence capable of modulating expression of a nucleic acid encoding a CRYO protein (such as SEQ ID NO 2, 4, 6, 8 or 10) in breeding programs. For example, in such a

program, a DNA marker is identified which may be genetically linked to the gene capable of modulating the activity of a protein of interest (for example SEQ ID NO 2, 4, 6, 8 or 10) in a plant (which gene can be the gene encoding a protein of interest or another gene capable of influencing the activity of a protein of interest). This DNA marker is then used in breeding programs to select plants having altered growth characteristics. Many techniques are nowadays available to identify SNPs and/or INDELs.

Also within the scope of the present invention are nucleic acids which are alternative splice variants of a CRYO protein encoded by any one of SEQ ID NO 1, 3, 5, 7 or 9. The term "alternative splice variant" as used herein encompasses variants of a nucleic acid encoding a CRYO protein in which introns and selected exons have been excised (for example, such that the mRNA has tissue-specific expression), optionally in response to specific signals. Such variants will be ones in which the biological activity of the protein remains unaffected, which can be achieved by selectively retaining functional segments of the protein. Methods for making such splice variants are well known in the art.

The invention furthermore encompasses nucleic acids that are capable of hybridising with a nucleic acid encoding a protein as represented by SEQ ID NO 2, 4, 6, 8 or 10. The term "hybridisation" as used herein is the process wherein substantially homologous complementary nucleotide sequences anneal to each other. The hybridisation process can occur entirely in solution, i.e. both complementary nucleic acids are in solution. Tools in molecular biology relying on such a process include the polymerase chain reaction (PCR; and all methods based thereon), subtractive hybridisation, random primer extension, nuclease S1 mapping, primer extension, reverse transcription, cDNA synthesis, differential display of RNAs, and DNA sequence determination. The hybridisation process can also occur with one of the complementary nucleic acids immobilised to a matrix such as magnetic beads, Sepharose beads or any other resin. Tools in molecular biology relying on such a process include the isolation of poly (A+) mRNA. The hybridisation process can furthermore occur with one of the complementary nucleic acids immobilised to a solid support such as a nitro-cellulose or nylon membrane or immobilised by e.g. photolithography to e.g. a siliceous glass support (the latter known as nucleic acid arrays or microarrays or as nucleic acid chips). Tools in molecular biology relying on such a process include RNA and DNA gel blot analysis, colony hybridisation, plaque hybridisation, in situ hybridisation and microarray hybridisation. In order to allow hybridisation to occur, the nucleic acid molecules are generally thermally or chemically denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridisation is influenced by conditions such as temperature, salt concentration and hybridisation buffer

composition. High stringency conditions for hybridisation include high temperature and/or low salt concentration (salts include NaCl and Na_3-citrate) and/or the inclusion of formamide in the hybridisation buffer and/or lowering the concentration of compounds such as SDS (detergent) in the hybridisation buffer and/or exclusion of compounds such as dextran sulphate or polyethylene glycol (promoting molecular crowding) from the hybridisation buffer. Conventional hybridisation conditions are described in, for example, Sambrook (2001) Molecular Cloning: a laboratory manual (3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York), but the skilled craftsman will appreciate that numerous different hybridisation conditions can be designed in function of the known or the expected homology and/or length of the nucleic acid. Typical conditions for "stringent hybridisation" are for example hybridising at a temperature of 60°C followed by washes in 2XSSC, 0.1XSDS, and 1X SSC, 0.1X SDS.

Advantageously, the method according to the present invention may also be practised using portions of a DNA or nucleic acid, which portions retain CRYO activity, i.e. a similar biological function to those encoding proteins represented in SEQ ID NO: 2, 4, 6, 8 or 10. Portions of a DNA sequence refer to a piece of DNA derived or prepared from an original (larger) DNA molecule, which DNA portion, when expressed in a plant, gives rise to plants having modified growth characteristics. The portion may comprise many genes, with or without additional control elements, or may contain just spacer sequences etc.

DNA sequences as defined in the current invention can also be interrupted by intervening sequences. With "intervening sequences" is meant any nucleic acid which disrupts a coding sequence in the DNA sequence of interest or which disrupts the expressible format of a DNA sequence comprising the DNA sequence of interest. Removal of intervening sequences restores the coding sequence or said expressible format. Examples of intervening sequences include introns and mobilisable DNA sequences such as transposons. With "mobilisable DNA sequence" is meant any DNA sequence that can be mobilized as the result of a recombination event.

The proteins encoded by the nucleic acids identified by the screening method according to the present invention were hitherto unknown. Therefore, the invention also provides an isolated protein selected from the group consisting of:

(a) a polypeptide as given in any one of SEQ ID NO 2, 4, 6, 8 or 10,

(b) a polypeptide encoded by a nucleic acid as defined above in any of (i) to (vi);

(c) a homologue, a derivative, an immunologically active and/or functional fragment of a protein as defined in (a) or (b).

Besides modifying tolerance to cold stress, the proteins may also be involved in protein transport and sorting (CRYO1 [SEQ ID NO 1/2], CRYO2 [SEQ ID NO 3/4], CRYO3 [SEQ ID NO 5/6] and CRYO4 [SEQ ID NO 7/8]), vacuole formation, development or functioning (CRYO1, CRYO2, CRYO3), in transcription and translation (CRYO5 [SEQ ID NO 9/10]), or in membrane fluidity (CRYO4).

Response to stress requires an adaptation of metabolism, including transport of proteins and other components between different organelles, in particular between the Golgi apparatus and the vacuole. Plant vacuoles perform different functions, depending on the cell type in which they occur. They play an important role in cell growth or function as storage organelles for proteins, ions, secondary metabolites and metabolic waste products. In this last aspect, vacuoles also resemble lysosomes. They contain many hydrolytic enzymes for degradation of damaged or redundant cell material. Adaptation to changing environmental conditions or to stress involves not only synthesis of new cellular components, but also degradation of cellular material. These degradation processes require an extensive trafficking of material via membrane bound vesicles such as endosomes. Also hydrolytic enzymes are delivered to the vacuole via endosomes. Certain mutants in yeast, known as "Class E" mutants (Jones *et al.*, In: Yeast III, Cold Spring Harbor Laboratory Press, p363 - 470, 1997), are unable to perform a correct sorting of proteins to the vacuole. Microscopical analysis reveals that these mutants contain large aberrant endosomal structures (Raymond *et al.*, Molecular Biology of the Cell 3, 1389, 1992), filled with proteins that are normally transited to the vacuole.

SEQ ID NO 2 (CRYO1) is a plant homologue of yeast SNF7 (=DID1 =VPS32 =YLR025W). SNF7 mutants belong to the group of class E vacuolar trafficking mutants (Jones *et al.*, In: Yeast III, Cold Spring Harbor Laboratory Press, p363 - 470, 1997). The SNF7 mutant accumulates a prominent organelle distinct from the vacuole, containing large amounts of enzymes which are normally present in the vacuole such as the hydrolases CpY, PrA & PrB. The protein is involved in derepression of SUC2 in response to glucose limitation. SNF7 mutants show a decrease in invertase derepression, a growth defect on raffinose, temperature-sensitive growth on glucose, and a sporulation defect in homozygous diploids. The SNF7 sugar related phenotype could be due to an altered turnover of a glucose sensor. These and other data suggest that the protein transport from the Golgi network and from the plasma membrane to the vacuole is interfered with. SNF7 forms a family of coiled-coil-forming proteins with vps20 and mos10. The proteins are involved in same trafficking step, endosome-to-vacuole transport, but probably participate in different cargo-specific events (Kranz et al., 2001).

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SEQ ID NO 4 (CRYO2) is an isoform of CRYO1.

SEQ ID NO 6 (CRYO3) is the plant homologue of yeast DID2 (=FT11 =YKR035W-A), another member of the class E vacuolar trafficking proteins. DID2 is related to SNF7; it has similar structural features, it may have a comparable function and possibly belongs to the same protein complex in yeast (Amerik *et al.*, Molecular Biology of the Cell, 11 3365 – 3380, 2000).

SEQ ID NO 8 (CRYO4) is a protein with homology to At1g72160, a cytosolic factor in *Arabidopsis thaliana*, and it has significant homology with yeast SEC14 (=YMR079W). This yeast protein is a cytosolic phosphatidylinositol/phosphatidylcholine transfer protein and is required for the transport of secretory proteins from the Golgi complex and for protein secretion (Bankaitis et al., 1989). In yeast it is associated with the Golgi complex as a peripheral membrane protein and forms a link between phospholipid metabolism and vesicle trafficking (Li et al., 2000).

SEQ ID NO 10 (CRYO5) codes for a protein with a RING-domain. RING-domain proteins are known to be involved in biological processes such as transcriptional and translational regulation, and in targeted proteolysis. The RING-domain mediates protein-protein interactions and is related to the zinc-finger domain.

"Homologues" of a protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes having amino acid substitutions, deletions and/or insertions relative to the unmodified protein in question and having similar biological and functional activity as the unmodified protein from which they are derived. To produce such homologues, amino acids of the protein may be replaced by other amino acids having similar properties (such as similar hydrophobicity, hydrophilicity, antigenicity, propensity to form or break α-helical structures or β-sheet structures). Conservative substitution tables are well known in the art (see for example Creighton (1984) Proteins. W.H. Freeman and Company). The homologues useful in the methods according to the invention have at least 50% sequence identity or similarity (functional identity) to the unmodified protein, alternatively at least 60% sequence identity or similarity to an unmodified protein, or alternatively at least 70% sequence identity or similarity to an unmodified protein. Typically, the homologues have at least 80% sequence identity or similarity to an unmodified protein, preferably at least 85% sequence identity or similarity, further preferably at least 90% sequence identity or similarity to an unmodified protein, most preferably at least 95% sequence identity or similarity to an unmodified protein.

Two special forms of homology, orthologous and paralogous, are evolutionary concepts used to describe ancestral relationships of genes. The term "parologous" relates to gene-duplications within the genome of a species leading to paralogous genes. The term "orthologous" relates to homologous genes in different organisms due to ancestral relationship. The term "homologues" as used herein also encompasses paralogues and orthologues of the proteins useful in the methods according to the invention.

Two polypeptides or nucleic acids are said to be "identical" if the sequence of amino acid residues or nucleotides, respectively, in the two sequences is the same when optimally aligned. Sequence comparisons between two (or more) polypeptide or nucleic acids are typically performed by comparing sequences of the two sequences over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (Adv. Appl. Math., **2** 482, 1981), by the homology alignment algorithm of Needleman and Wunsch (J. Mol. Biol., **48** 443, 1970), by the search for similarity method of Pearson and Lipman (PNAS, **85** 2444, 1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

The term "derivatives" refers to peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise substitutions, deletions or additions of naturally and non-naturally occurring amino acid residues compared to the amino acid of a naturally-occurring form of the proteins as presented in SEQ ID NO 2, 4, 6, 8 or 10. "Derivatives" of a CRYO protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise naturally occurring altered, glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid of a naturally-occurring form of the polypeptide. A derivative may also comprise one or more non-amino acid substituents compared to the amino acid from which it is derived, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid such as, for example, a reporter molecule which is bound to facilitate its detection, and non-naturally occurring amino acid residues relative to the amino acid of a naturally-occurring protein. "Substitutional variants" of a protein are those in which at least one residue in an amino acid has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered

depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues, and deletions will range from about 1-20 residues. Preferably, amino acid substitutions comprise conservative amino acid substitutions. "Insertional variants" of a protein are those in which one or more amino acid residues are introduced into a predetermined site in said protein. Insertions can comprise amino-terminal and/or carboxy-terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid will be smaller than amino- or carboxy-terminal fusions, in the order of about 1 to 10 residues. Examples of amino- or carboxy-terminal fusion proteins or peptides include the binding domain or activation domain of a transcriptional activator as used in the yeast two-hybrid system, phage coat proteins, $(histidine)_6$-tag, glutathione S-transferase-tag, protein A, maltose-binding protein, dihydrofolate reductase, Tag·100 epitope, c-myc epitope, FLAG®-epitope, lacZ, CMP (calmodulin-binding peptide), HA epitope, protein C epitope and VSV epitope. "Deletion variants" of a protein are characterised by the removal of one or more amino acids from the protein.

Amino acid variants of a protein may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce substitution, insertion or deletion variants of a protein are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA are well known to those skilled in the art and include M13 mutagenesis, T7-Gen *in vitro* mutagenesis (USB, Cleveland, OH), QuickChange Site Directed mutagenesis (Stratagene, San Diego, CA), PCR-mediated site-directed mutagenesis or other site-directed mutagenesis protocols.

"Active fragments" of a CRYO protein encompasses at least five contiguous amino acid residues of a protein, in case of a functional fragment the minimum size being a sequence of sufficient size to provide this sequence with at least a comparable function and/or activity to the original sequence which was truncated, while the maximum size is not critical. Typically, the truncated amino acid will range from about 5 to about 60 amino acids in length. "Immunologically active" refers to molecules or specific fragments thereof, such as specific epitopes or haptens, that are recognised by (i.e. that bind to) antibodies. Specific epitopes may be determined using, for example, peptide-scanning techniques as described in Geysen *et al.*, Chem Biol., 3 (8), 679-688, 1996. Functional fragments can also include those comprising an epitope which is specific for the proteins according to the invention.

The present invention also relates to a recombinant genetic construct comprising a nucleic acid according to the invention. The genetic constructs facilitate the introduction and/or

expression and/or maintenance of a nucleotide sequence as defined above into a plant cell, tissue or organ. Preferably, the genetic construct comprises:

(i) an isolated nucleic acid obtainable by the screening method as outlined above, preferably a nucleic acid sequence encoding a protein as defined in any of (i) to (vi) above;

(ii) a regulatory element operably linked to the nucleic acid of (i), which regulatory element is preferably a plant expressible promoter; and optionally

(iii) a transcription termination sequence.

The nucleic acid construct can be an expression vector wherein the nucleic acid is operably linked to one or more regulatory elements allowing expression in prokaryotic and/or eukaryotic host cells. The vector may be commercially available, suitable for transforming into plants and suitable for expression of the gene of interest in the transformed cells.

Advantageously, any nucleic acid obtainable by the screening method according to the present invention can be used in the construct; preferably a nucleic acid as defined in any of (i) to (vi) above is used.

The term "operably linked" as used herein refers to a functional linkage between the regulatory element and the gene of interest, such that the regulatory element is able to initiate transcription of the gene of interest.

As used herein, the term "plant-expressible promoter" refers to a promoter that is capable of driving transcription in a plant cell. This not only includes any promoter of plant origin, such as the natural promoter of the transcribed DNA sequence, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell. The promoter may also be an artificial or synthetic promoter. The term "plant-expressible promoter" includes, but is not restricted to, constitutive, inducible, organ-, tissue- or cell-specific and/or developmentally regulated promoters. The terms "regulatory element", "control sequence", "promoter" are all used herein interchangeably and, taken in a broad context, refer to regulatory nucleic acids capable of effecting expression of the sequences to which they are ligated.

Advantageously, any type of promoter may be used to drive expression of the nucleic acid encoding a CRYO protein. More specifically, a constitutive promoter can be, but is not restricted to, one of the following: a 35S promoter (Odell *et al.*, Nature, **313** 482 - 493, 1985), a 35S'3 promoter (Hull and Howell, Virology, **86** 482 – 493, 1987), the promoter of the nopaline synthase gene ("PNOS") of the Ti-plasmid (Herrera-Estrella, Nature, **303** 209 - 213, 1983) or

the promoter of the octopine synthase gene ("POCS", De Greve *et al.*, J. Mol. Appl. Genet. 1 (6), 499 - 511, 1982). It is clear that other constitutive promoters can be used to obtain similar effects. A meristem-specific promoter, such as the rnr (ribonucleotide reductase), cdc2a promoter and the cyc07 promoter, could be used to effect expression in all growing parts of the plant, thereby increasing cell proliferation, which in turn would increase yield or biomass. If the desired outcome would be to influence seed characteristics, such as the storage capacity, seed size, seed number, biomass etc., then a seed-specific promoter, such as p2S2, pPROLAMIN, pOLEOSIN could be selected. An aleurone-specific promoter may be selected in order to increase growth at the moment of germination, thereby increasing the transport of sugars to the embryo. An inflorescence-specific promoter, such as pLEAFY, may be utilised if the desired outcome would be to modify the number of flower organs. To produce male-sterile plants one would need an anther specific promoter. To impact on flower architecture for example petal size, one could choose a petal-specific promoter. If the desired outcome would be to modify growth and/or developmental characteristics in particular organs, then the choice of the promoter would depend on the organ to be modified. For example, use of a root-specific promoter would lead to increased growth and/or increased biomass or yield of the root and/or phenotypic alteration of the root. This would be particularly important where it is the root itself that is the desired end product, such crops include sugar beet, turnip, carrot, and potato. A fruit-specific promoter may be used to modify, for example, the strength of the outer skin of the fruit or to increase the size of the fruit. A green tissue-specific promoter may be used to increase leaf size. A cell wall-specific promoter may be used to increase the rigidity of the cell wall, thereby increasing pathogen resistance. An anther-specific promoter may be used to produce male-sterile plants. A vascular-specific promoter may be used to increase transport from leaves to seeds. A nodule-specific promoter may be used to increase the nitrogen fixing capabilities of a plant, thereby increasing the nutrient levels in a plant. A stress-inducible promoter may also be used to drive expression of a nucleic acid to increase membrane integrity during conditions of stress. A stress inducible promoter such as the water stress induced promoter WSI18, the drought stress induced Trg-31 promoter, the ABA related promoter rab21 or any other promoter which is induced under a particular stress condition such as temperature stress (cold, freezing, heat) or osmotic stress, or drought stress or oxidative stress or biotic stress can be used to drive expression of a CRYO gene.

If the desired outcome would be to influence the cold tolerance of a plant under adverse conditions, then a cold-inducible promoter such as, for example, prd29, pws18 or pcor15 could be selected.

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Suitable promoters for expression in yeast are known in the art, see for example *Current Protocols in Molecular Biology*, Unit 13 (Ausubel *et al.*, 1994) and the *Guide to Yeast Genetics and Molecular Biology* (Guthrie and Fink, 1991).

The recombinant genetic construct according to the present invention may include further regulatory or other sequences from other genes. Encompassed are transcriptional regulatory sequences derived from a classical eukaryotic genomic gene (including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence) and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. Also included is a transcriptional regulatory sequence of a classical prokaryotic gene, in which case it may include a –35 box sequence and/or –10 box transcriptional regulatory sequences. Regulatory elements also encompass a synthetic fusion molecule or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

Optionally, one or more terminator sequences may also be used in the construct introduced into a plant. The term "terminator" encompasses a control sequence which is a DNA sequence, at the end of a transcriptional unit, which signals 3' processing and polyadenylation of a primary transcript and termination of transcription. Additional regulatory elements may include transcriptional as well as translational enhancers. Those skilled in the art will be aware of terminator and enhancer sequences which may be suitable for use in performing the invention.

Furthermore, the recombinant nucleic acid can be constructed and employed to target the gene product of the nucleic acid of the invention to a specific intracellular compartment within a plant cell or to direct a protein to the extracellular environment. This can generally be obtained by operably joining a DNA sequence encoding a transit or signal peptide to the recombinant nucleic acid.

The genetic constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type, for example a bacterial cell, when the genetic construct is required to be maintained as an episomal genetic element (e.g. plasmid or cosmid molecule) in a cell. Preferred origins of replication include, but are not limited to, the f1-ori and colE1 origins of replication.

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The genetic construct may optionally comprise a selectable marker gene. As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof. Suitable markers may be selected from markers that confer antibiotic or herbicide resistance. Cells containing the recombinant DNA will thus be able to survive in the presence of antibiotic or herbicide concentrations that kill untransformed cells. Examples of selectable marker genes include the bar gene which provides resistance to the herbicide Basta; the ampicillin resistance gene (Amp^r), the tetracycline resistance gene (Tc^r), the bacterial kanamycin resistance gene (Kan^r), the phosphinothricin resistance gene, the neomycin phosphotransferase gene (nptII), the hygromycin resistance gene, and the chloramphenicol acetyltransferase (CAT) gene. Visual markers, such as the Green Fluorescent Protein (GFP, Haseloff *et al.*, Nature **334**, 585 – 591, 1997), β-glucuronidase (GUS), and luciferase, may also be used as selectable markers.

According to another embodiment, the present invention relates to the use of the nucleic acids of the present invention as selectable marker gene in plants or other organisms. More preferably, the present invention also relates to the use of a gene coding for a CRYO protein as defined above as selectable marker gene, selection taking place by treating with a stress condition such as a sub-optimal growth temperature.

The nucleic acids obtainable by the screening method as described herein encode proteins that support faster growth of yeast under stress conditions, therefore it is likely, since these nucleic acids originate from plants, that modulation of expression of these nucleic acids upon introduction into plants, will also support faster growth of plants under stress conditions. Therefore the present invention provides a method for increasing stress tolerance of plants, preferably to cold stress, comprising modulating expression in plants of a nucleic acid sequence encoding a CRYO protein and/or modulating activity of a CRYO protein. Similarly, the present invention provides a method for increasing stress tolerance of yeast, preferably to cold stress, comprising modulating expression in plants of a nucleic acid sequence encoding a CRYO protein and/or modulating activity of a CRYO protein.

"Altered stress tolerance" as used herein comprises, for any given stress, increasing tolerance in plants or yeast to that particular stress, whether those plants or yeast already have some degree of tolerance to the particular stress or whether that plant or yeast is being provided with tolerance to that stress anew.

Preferably, the altered tolerance is to at least one of temperature stress, osmotic stress, drought stress or oxidative stress, more preferably cold stress.

The terms "tolerance" and "resistance" as used herein encompass protection against stress ranging from a delay to substantially a complete inhibition of alteration in cellular metabolism, reduced cell growth and/or cell death caused by environmental stress conditions. Advantageously, transgenic plants or yeasts obtained by the methods of the present invention are tolerant or resistant to environmental stress conditions.

The term "environmental stress" as used herein encompasses stress factors such as drought stress (water, dehydration), osmotic stress, salt stress, temperature stress (due to for example heat or frost). "Temperature stress" which includes "cold stress", "freezing stress" or "heat stress" is a stress induced by sub-optimal or supra-optimal growth temperatures for a particular organism. Optimal growth temperature ranges may be readily determined or would be known to those skilled in the art. "Osmotic stress" is any stress associated with or induced by loss of water, reduced turgor or reduced water content of a cell, tissue, organ or whole plant. "Drought stress" refers to any stress which is induced by or associated with the deprivation of water or reduced supply of water to a cell, tissue, organ or organism. The term "salt-stress" refers to any stress which is associated with or induced by elevated concentrations of salt and which result in a perturbation in the osmotic potential of the intracellular or extracellular environment of a cell. "Oxidative stress" occurs in situations of cold stress combined with intensive light, in situations of ozone stress, in cases of necrosis as a result of pathogen infection or wounding, in cases of senescence and due to application of certain herbicides (like atrazine or paraquat).

According to a preferred feature of the invention, the stress is cold stress. Advantageously the results of testing for tolerance or resistance to environmental conditions in the yeast cells give a reliable measure of the capability of the inserted coding sequence or gene to induce tolerance or resistance to environmental stress in plants. The capacity of an isolated nucleic acid to confer tolerance or resistance to environmental stress tolerance to plants can be tested according to methods well-known in the art, see for example, Physical Stresses in Plants: Genes and Their Products for Tolerance. S. Grillo (Editor), A. Leone (Editor) (June 1996), Springer Verlag; ISBN: 3540613471; Handbook of Plant and Crop Stress. Mohammad Peassarakli (Editor), Marcel Dekker, ISBN: 0824789873; The Physiology of Plants Under Stress; Abiotic Factors. Erik T. Nilsen, David M. Orcutt (Contributor), Eric T. Nilsen. 2nd edition (October 1996), John Wiley & Sons; ISBN: 047131526; Drought, Salt, Cold and Heat Stress: Molecular Responses in Higher Plants (Biotechnology Intelligence Unit). Kazuo Shinozaki (Editor), Kazuko Yamaguchi-Shinozaki (Editor) (1999). R G Landes Co; ISBN: 1570595631;

Plants Under Stress: Biochemistry, Physiology and Ecology and Their Application to Plant Improvement (Society for Experimental Biology Seminar Serie). Hamlyn G. Jones, T.J. Flowers, M.B. Jones (Editor). (September 1989). Cambridge Univ. Pr. (Short); ISBN: 0521344239; Plant Adaptation to Environmental Stress. Leslie Fowden, Terry Mansfield, John Stoddart (Editor) (October 1993) Chapman & Hall; ISBN: 0412490005; or the appended examples. Similar methods exist for yeast; see for example: The molecular and cellular biology of the yeast Saccharomyces cerevisiae. Pringle, Jones, Broach and Strathern, Cols Spring Harbor laboratory press, 1992 (New York); Guide to yeast Genetics and Mollecular and Cell Biology (Volum 350 and 351 of Methods in enzymology) (Guthrie and Finf Eds), Academic Press (2002) San Diego; Yeast Gene Analysis (Brown and Tuite) (Volume 26 of Methods in Microbiology) Academic press (San Diego); Yeast Stress Responses (Ed. Hohmann and Mager) Springer Verlag, Heidelberg 1997.

Modulation (enhancing or decreasing) of expression of a nucleic acid encoding a CRYO protein or modulation of a CRYO protein itself encompasses altered expression of a gene or altered levels of a gene product, namely a polypeptide, in specific cells or tissues, which gene or gene-product influences CRYO gene expression or protein activity.

The nucleic acids obtained by the screening method according to the invention will have the capacity to modify tolerance to cold stress in plants or yeast. This effect may also be obtained by applying the proteins encoded by the nucleic acids as defined above, directly to the plants or yeast.

Preferably, modulation of expression of a nucleic acid encoding a CRYO protein and/or modulation of activity of the CRYO protein itself is effected by recombinant means. Such recombinant means may comprise a direct and/or indirect approach for modulation of expression of a nucleic acid and/or for modulation of the activity of a protein.

For example, an indirect approach may comprise introducing, into a plant, a nucleic acid capable of modulating activity of the protein in question (a CRYO protein) and/or expression of the gene in question (a gene encoding a CRYO protein). The CRYO gene or the CRYO protein may be wild type, i.e. the native or endogenous nucleic acid or polypeptide. Alternatively, it may be a nucleic acid derived from the same or another species, which gene is introduced as a transgene, for example by transformation. This transgene may be substantially modified from its native form in composition and/or genomic environment through deliberate human manipulation. Also encompassed by an indirect approach for modulating activity of a CRYO protein and/or expression of a CRYO gene is the inhibition or stimulation of regulatory

sequences that drive expression of the native gene or transgene. Such regulatory sequences may be introduced into a plant.

A direct and more preferred approach comprises introducing into a plant or yeast a nucleic acid encoding a CRYO protein or a homologue, derivative or active fragment thereof. The nucleic acid may be introduced by, for example, transformation. The nucleic acid may be derived (either directly or indirectly (if subsequently modified)) from any source provided that the sequence, when expressed in a plant or yeast, leads to modulated expression of a CRYO nucleic acid/gene or modulated activity of a CRYO protein.

Preferably, the nucleic acid is isolated from a halophytic plant, more preferably from *Beta vulgaris*. Most preferably, the nucleic acid capable of modulating expression of a CRYO gene or activity of a CRYO protein in a plant is a nucleic acid as represented by SEQ ID NO 1, 3, 5, 7, 9, or homologues, derivatives or active fragments thereof, or a nucleic acid encoding a protein represented by SEQ ID NO 2, 4, 6, 8 or 10, or homologues, derivatives or active fragments thereof.

However, it should be clear that the applicability of the invention is not limited to use of a nucleic acid represented by SEQ ID NO 1, 3, 5, 7, 9 nor to the nucleic acid encoding the protein of SEQ ID NO 2, 4, 6, 8 or 10, but that other nucleic acids encoding homologues, derivatives or active fragments of SEQ ID NO 1 to 10 may be useful in the methods of the present invention. Advantageously, the method according to the present invention serves to confer tolerance or resistance to environmental stress conditions in plants and parts thereof, or in yeast.

Modulating the activity of a nucleic acid/gene can be achieved for example by inhibiting or stimulating control elements that drive expression of a native gene or of a transgene, such regulatory sequences may be introduced into a plant or yeast. The "nucleic acid" or "protein" may be wild type, i.e. the native or endogenous nucleic acid or polypeptide. Alternatively, the gene may be a heterologous nucleic acid derived from the same or another species, which gene is introduced as a transgene, for example, by transformation. This transgene may be substantially modified from its native form in composition and/or genomic environment through deliberate human manipulation. Modulating gene expression also encompasses altered transcript level of a gene, which can be sufficient to induce certain phenotypic effects.

According to a preferred feature of the present invention, enhanced or increased expression of a nucleic acid is envisaged. Methods for obtaining enhanced or increased expression of genes

or gene products are well documented in the art and include, for example, overexpression driven by a strong promoter, the use of transcription enhancers or translation enhancers.

However downregulation of the expression of a nucleic acid may also give rise to modified stress tolerance in a plant or yeast. Advantageously, plants having modified stress tolerance may be obtained by expressing a nucleic acid encoding a CRYO protein in either sense or antisense orientation. Techniques for downregulation are well known in the art. Similar and other approaches for downregulation expression in yeast are known in the art (for example interruption of the ORF with a gene complementing a metabolic defect of the host strain or with a gene from bacteria conferring tolerance to the antibiotics Kanamycin or Genetycin).

Another embodiment of the invention provides host cells comprising a nucleic acid molecule encoding a CRYO protein. Preferred host cells are plant cells or yeast. The polypeptides of the present invention may also be produced by recombinant expression in prokaryotic and eukaryotic engineered cells, other than plant cells, such as bacteria, fungi, or animal cells. Suitable expression systems are known to those skilled in the art.

The invention extends to plants or yeast tolerant to environmental stress, preferably cold stress, which plants or yeast have elevated levels of a protein as defined above. The present invention thus also encompasses plants obtainable by the methods according to the present invention. The present invention therefore provides plants obtainable by the method according to the present invention, which plants have modified stress tolerance and which plants have altered CRYO protein activity and/or altered expression of a nucleic acid encoding a CRYO protein.

The present invention also relates to a method for the production of transgenic plants, plant cells or plant tissues, comprising introduction of a nucleic acid molecule of the invention in an expressible format or a genetic construct as defined above into a plant, plant cell or plant tissue. Therefore, according to a fifth embodiment of the present invention there is provided a method for producing transgenic plants having modified tolerance to stress, relative to corresponding wild type plants, which method comprises:

(i) introducing into a plant cell a nucleic acid encoding a CRYO protein; and

(ii) cultivating this plant cell under conditions promoting regeneration and mature plant growth.

Preferably, the stress is at least one of cold stress, salt stress, osmotic stress, drought stress or oxidative stress. More preferably, the stress is cold stress.

The present invention extends to any plant cell, plant or plant part or yeast cell obtained by any of the methods described herein, and to all plant parts, including harvestable parts of a plant, and propagules thereof. The present invention also encompasses a plant or a part thereof comprising a plant cell transformed with a nucleic acid according to the invention. The present invention extends further to encompass the progeny of a primary transformed or transfected cell, tissue, organ or whole plant that has been produced by any of the aforementioned methods, the only requirement being that progeny exhibit the same genotypic and/or phenotypic characteristic(s) as those produced in the parent by the methods according to the invention.

The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants, plant parts, plant cells, tissues and organs. The term "plant" also therefore encompasses suspension cultures, embryos, meristematic regions, callus tissue, leaves, flowers, fruits, seeds, rhizomes, bulbs, roots (including tubers), shoots (including stem cultures), gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily *Viridiplantae*, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising Acanthaceae, Aceraceae, Acoraceae, Adiantaceae, Agavaceae, Aizoaceae, Alismataceae, Alliaceae, Aloeaceae, Alstroemeriaceae, Amaranthaceae, Amaryllidaceae, Anacardiaceae, Anemiaceae, Angiopteridaceae, Annonaceae, Apocynaceae, Aponogetonaceae, Aquifoliaceae, Araceae, Araliaceae, Araucariaceae, Arecaceae, Aristolochiaceae, Asparagaceae, Aspleniaceae, Asteliaceae, Asteraceae, Balsaminaceae, Basellaceae, Bataceae, Begoniaceae, Berberidaceae, Betulaceae, Bignoniaceae, Bixaceae, Blechnaceae, Bombacaceae, Boraginaceae, Brassicaceae: *Alliaria petiolata, Arabidopsis thaliana, Arabis petiolaris, Arabis pumila,* Arabis sp., *Berteroa incana, Biscutella laevigata, Brassicajunceae, Brassica napus, Brassica napus* var. *napus, Brassica nigra, Brassica oleracea, Brassica oleracea* var. *gongylo, Capsella bursa-pastoris, Cardamine pratensis, Cochlearia officinalis, Dentaria laciniata, Descurainia pinnata, Draba asprella, Draba verna, Draba, Erysimum asperum, Erysimum asperum, Erysimum capitatum, Lepidiumflavum, Lepidium virginicum, Lesquerella argyraea, Lesquerella densiflora, Lesquerella rubicundula,* Lesquerella sp., *Lobularia maritima, Lunaria annua, Lunaria rediviva, Neobeckia aquatica, Nerisyrenia camporum, Physaria chambersii, Raphanus sativus, Sinapis alba, Stanleya pinnata, Streptanthus cordatus, Thlaspi arvense, Thlaspi rotundifolium,* Bromeliaceae, Buddlejaceae, Burseraceae, Buxaceae, Cabombaceae, Cactaceae, Caesalpiniaceae, Callitrichaceae, Calochortaceae, Calyceraceae, Campanulaceae, Cannabaceae, Cannaceae, Capparaceae, Caprifoliaceae, Caricaceae, Caryophyllaceae, Casuarinaceae, Celastraceae,

Chenopodiaceae, Cistaceae, Clusiaceae, Cneoraceae, Cochlospermaceae, Combretaceae, Commelinaceae, Convallariaceae, Convolvulaceae, Comaceae, Corylaceae, Crassulaceae, Crossosomataceae, Cucurbitaceae, Cunoniaceae, Cupressaceae, Cuscutaceae, Cyatheaceae, Cycadaceae, Cyperaceae, Cyrillaceae, Dennstaedtiaceae, Dicksoniaceae, Didiereaceae, Dilleniaceae, Dioscoreaceae, Dipsacaceae, Dipterocarpaceae, Droseraceae, Dryopteridaceae, Ebenaceae, Ehretiaceae, Elaeagnaceae, Elaeocarpaceae, Elatinaceae, Empetraceae, Epacridaceae, Ephedraceae, Equisetaceae, Ericaceae, Eriocaulaceae, Erythroxylaceae, Escalloniaceae, Euphorbiaceae, Eupomatiaceae, Fabaceae, Fagaceae, Flacourtiaceae, Fouquieriaceae, Frankeniaceae, Fumariaceae, Gentianaceae, Geraniaceae, Gesneriaceae, Ginkgoaceae, Globulariaceae, Goodeniaceae, Grossulariaceae, Gunneraceae, Haemodoraceae, Haloragaceae, Hamamelidaceae, Heliconiaceae, Hippocastanaceae, Hyacinthaceae, Hydrangeaceae, Hydrophyllaceae, Hypericaceae, Iridaceae, Isoetaceae, Juglandaceae, Juncaceae, Koeberliniaceae, Krameriaceae, Lamiaceae, Lauraceae, Lecythidaceae, Lemnaceae, Lentibulariaceae, Liliaceae, Limnanthaceae, Limnocharitaceae, Linaceae, Loasaceae, Lobeliaceae, Loganiaceae, Lomandraceae, Lomariopsidaceae, Loranthaceae, Lycopodiaceae, Lythraceae, Magnoliaceae, Malpighiaceae, Malvaceae, Marantaceae, Marcgraviaceae, Marsileaceae, Martyniaceae, Mayacaceae, Melanthiaceae, Melastomataceae, Meliaceae, Melianthaceae, Menispermaceae, Menyanthaceae, Mimosaceae, Monimiaceae, Monotropaceae, Moraceae, Musaceae, Myoporaceae, Myricaceae, Myristicaceae, Myrsinaceae, Myrtaceae, Nelumbonaceae, Nyctaginaceae, Nymphaeaceae, Nyssaceae, Ochnaceae, Oenotheraceae, Oleaceae, Oliniaceae, Onagraceae, Ophioglossaceae, Orchidaceae, Orobanchaceae, Osmundaceae, Oxalidaceae, Paeoniaceae, Pandanaceae, Papaveraceae,Passifloraceae, Pedaliaceae, Phillydraceae, Phormiaceae, Phytolaccaceae, Pinaceae, Piperaceae, Pittosporaceae, plantaginaceae, Platanaceae, Plumbaginaceae, Poaceae, Podocarpaceae, Podophyllaceae, Polemoniaceae, Polygalaceae, Polygonaceae, Polypodiaceae, Pontederiaceae, Portulacaceae, Primulaceae, Proteaceae, Pteridaceae, Punicaceae, Pyrolaceae, Rafflesiaceae, Ranunculaceae, Resedaceae, Restionaceae, Rhamnaceae, Rosaceae, Rubiaceae, Ruscaceae, Rutaceae, Salicaceae, Salviniaceae, Santalaceae, Sapindaceae, Sapotaceae, Sarraceniaceae, Saururaceae, Saxifragaceae, Scrophulariaceae, Selaginellaceae, Simaroubaceae, Smilacaceae, Solanaceae, Sparganiaceae, Sterculiaceae, Strelitziaceae, Styracaceae, Taccaceae, Tamaricaceae, Taxaceae, Taxodiaceae, Theaceae, Thelypteridaceae, Thymelaeaceae, Tiliaceae, Trapaceae, Tremandraceae, Trilliaceae, Trochodendraceae, Tropaeolaceae, Tumeraceae, Typhaceae, Ulmaceae, Urticaceae, Valerianaceae, Verbenaceae, Veronicaceae, Violaceae, Viscaceae, Vitaceae, Welwitschiaceae, Winteraceae, Xanthorrhoeaceae, Xerophyllaceae, Xyridaceae, Zamiaceae, Zingiberaceae, and Zygophyllaceae. According to a preferred feature of the present invention, the plant is a

monocotyledonous or dicotyledonous plant, such as a crop plant selected from rice, maize, wheat, barley, soybean, sunflower, canola, alfalfa, millet, barley, rapeseed and cotton. Additional species such as amaranth, artichoke, asparagus, broccoli, Brussels sprouts, cabbage, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, sugar beet, sugar cane, tomato, squash, and tea, trees and algae are not excluded. Further advantageously, plants obtained by the methods according to the invention enable crops to be grown with improved yield, growth, development and productivity under stress conditions, preferably under conditions of cold stress. The present invention also enables crops to be grown in areas which would otherwise not be possible.

The gene of interest is preferably introduced into a plant by transformation. The term "transformation" as referred to herein encompasses the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for transfer. The polynucleotide may be transiently or stably introduced into a host cell and may be maintained non-integrated, for example, as a plasmid, or alternatively, may be integrated into the host genome. The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known to persons skilled in the art. Transformation of a plant species is now a fairly routine technique. Advantageously, any of several transformation methods may be used to introduce the gene of interest into a suitable ancestor cell. Transformation methods include the use of liposomes, electroporation, chemicals that increase free DNA uptake, injection of the DNA directly into the plant, particle gun bombardment, transformation using viruses or pollen and microprojection. Methods may be selected from the calcium/polyethylene glycol method for protoplasts (Krens *et al.*, Nature, **296** 72 – 74, 1982; Negrutiu I. *et al.*, Plant Mol. Biol., **8** 363 - 373, 1987); electroporation of protoplasts (Shillito *et al.*, Bio/Technol, **3** 1099 - 1102, 1985); microinjection into plant material (Crossway *et al.*, Mol. Gen Genet, **202** 179 - 185, 1986); DNA or RNA-coated particle bombardment (Klein *et al.*, Nature, **327** 70 1987) infection with (non-integrative) viruses and the like, *Agrobacterium*-mediated transformation (Cheng *et al.* 1997 – WO 97/48814; Hansen 1998 – WO 98/54961, Hiei *et al.* 1994 – WO 94/00977; Hiei *et al.* 1998 – WO 98/17813; Rikiishi *et al.* 1999 – WO 99/04618; Saito *et al.* 1995 – WO 95/06722), including the 'flower dip' transformation method (Bechtold and Pelletier, Methods Mol. Biol., **82** 259 – 266, 1998; Trieu *et al.*, Plant J., **22** (6) 531 – 541, 2000).

Generally after transformation, plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant-expressible genes co-transferred with the gene of interest, following which the transformed material is regenerated into a whole plant. A whole organism may be regenerated from a single transformed or transfected cell, using methods known in the art. Plant tissue capable of subsequent clonal propagation, whether by

organogenesis or embryogenesis, may be transformed with a genetic construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the gene of interest, copy number and/or genomic organisation. Alternatively or additionally, expression levels of the newly introduced DNA may be undertaken using Northern and/or Western analysis, both techniques being well known to persons having ordinary skill in the art.

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformants, and the T2 plants further propagated through classical breeding techniques.

The generated transformed organisms may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed rootstock grafted to an untransformed scion).

Furthermore, the invention also relates to the use of a nucleic acid encoding a CRYO protein or of a CRYO protein itself, to modify stress tolerance of plants or parts thereof or of plant cells. The sequences as depicted in SEQ ID NO 1 to SEQ ID NO 10 are revealed to be involved in important processes leading to stress tolerance, as exemplified by plants having altered stress tolerance, which plants have been transformed with a sequence essentially similar to SEQ ID NO 1, 3, 5, 7 or 9. Similarly, the invention also relates to the use of a nucleic acid encoding a CRYO protein or of a CRYO protein itself, to modify stress tolerance of yeast. Preferably, the stress is at least one of temperature stress, osmotic stress, drought stress or oxidative stress.

Furthermore, the characteristic of the transgenic plants of the present invention to display tolerance to cold stress conditions can be combined with other approaches to confer cold stress tolerance to plants, e.g., use of osmotic protectants such as mannitol, proline; glycine-

betaine, water-channeling proteins, etc. Thus, the approach of the present invention to confer tolerance to environmental stress conditions to plants can be combined with known approaches which include introduction of various stress tolerance genes. Combination of these approaches may have additive and/or synergistic effects in enhancing tolerance or resistance to environmental stress.

The methods of the present invention to create plants with enhanced tolerance to stress can also be combined with other traits of interest, for example:

(i) herbicide tolerance (DE-A 3701623; Stalker, Science 242 (1988), 419),

(ii) insect resistance (Vaek, Plant Cell 5 (1987), 159-169),

(iii) virus resistance (Powell, Science 232 (1986), 738-743; Pappu, World Journal of Microbiology & Biotechnology 11 (1995), 426-437; Lawson, Phytopathology 86 (1996) 56 suppl.),

(iv) ozone resistance (Van Camp, Biotech. 12 (1994), 165-168),

(v) improving the preserving of fruits (Oeller, Science 254 (1991), 437-439),

(vi) improvement of starch composition and/or production (Stark, Science 242 (1992), 419; Visser, Mol. Gen. Genet. 225 (1991), 289-296),

(vii) altering lipid composition (Voelker, Science 257 (1992), 72-74),

(viii) production of (bio)polymers (Poirer, Science 256 (1992), 520-523),

(ix) alteration of the flower colour, e.g., by manipulating the anthocyanin and flavonoid biosynthetic pathway (Meyer, Nature 330 (1987), 667-678, WO90/12084),

(x) resistance to bacteria, insects and fungi (Duering, Molecular Breeding 2 (1996), 297-305; Strittmatter, Bio/Technology 13 (1995), 1085-1089; Estruch, Nature Biotechnology 15 (1997), 137-141),

(xi) alteration of alkaloid and/or cardia glycoside composition,

(xii) inducing maintaining male and/or female sterility (EP-A1 0 412 006; EP-A1 0 223 399; WO93/25695);

(xiii) higher longevity of the inflorescences/flowers, and

(xiv) abiotic stress resistance, other than temperature stress

Description of the figures:

The present invention will now be illustrated with reference to the following figures:

Fig.1: Cold sensitivity of the wild type (wt) yeast strain compared to the *gpd1* mutant. The yeast cells were grown on YPD (top row) or on SD medium (bottom row) at 30°C (control, left column), at 10°C (middle column) or at 15 °C (top right). The WT strain showed reduced growth compared to the gpd1 strain.

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Fig.2: Cold tolerance of the wild type yeast strain transformed with the CRYO1, CRYO2, CRYO3, CRYO4 or CRYO5 gene, compared to the wt yeast strain transformed with an empty vector (pYPGE@). (a) Enhanced growth after 10 days at 10°C of the yeast cells transformed with the CRYO1, CRYO2 or CRYO3 genes, or after 14 days for yeast cells transformed with the CRYO4 gene. (b) Enhanced growth of the yeast cells transformed with the CRYO5 gene compared to wild type yeast transformed with an empty vector (pYPGE). The two left panels are controls grown at 30°C on YPD medium or SD medium. The two right panels show growth of the same yeast strains grown at 10°C on YPD medium or SD medium.

Fig.3: Alignment between sequences of CRYO1 and CRYO2 from sugar beet and their homologues in *Arabidopsis* and yeast. At= *Arabidopsis thaliana*, Bv= *Beta vulgaris* and Sc= *Saccharomyces cerevisiae*.

Fig.4: Alignment between the sequences of CRYO3 and homologous proteins from various organisms, showing a high degree of conservation among the different species. At= *Arabidopsis thaliana*; *Bv= Beta vulgaris*; *Mm= Mus musculus*; *Hs= Homo sapiens*; *Sp= Schizosaccharomyces pombe*.

Fig. 5: Southern blot with a *CRYO1* and *CRYO2* probe on genomic sugar beet DNA. Enzymes used were *Bam*HI, *Hind*III and *Eco*RI.

Fig. 6: a) Northern blot with a *CRYO2* probe. Different timepoints (in hrs) after treating the sugar beet plants with 250 mM NaCl are indicated. α_3-tubulin was used as control. b) Northern blot with a *CRYO2* probe. Different timepoints (in hrs) after treating the sugar beet plants with 100 µM ABA are indicated. α_3-tubulin was used as control.

Fig.7: Growth of wild type yeast (upper row) and yeast transformed with the *CRYO5* gene (bottom row) on YPD (left panel) and YPD supplemented with 1 mM tert-butyl hydroperoxide (right panel).

Examples

Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY and in volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular

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Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd. (UK) and Blackwell Scientific Publications, UK.

Example 1: Construction of a sugar beet cDNA library induced by salt stress:

Sugar beet seeds (*Beta vulgaris* cv. Dita) were sown in pots containing a mixture of sand and vermiculite (1:1 w/w). The plants were grown under greenhouse conditions (8 h at 20°C, 16 h at 25°C with supplementary lighting to ensure a minimum of 12 h photoperiod). The plants were periodically irrigated with a nutrient solution (2.4 g/l $Ca(NO3)_2 \cdot 4H_2O$, 1 g/l KNO_3, 1 g/l $MgSO_4 \cdot 7H_2O$, 0.3 g/l KH_2PO_4, 5.6 mg/l Fequelate (Kelantren, Bayer), 1.1 mg/l $ZnSO_4 \cdot 7H_2O$, 3.3 mg/l $MnO_4 \cdot H_2O$, 0.3 mg/l $CuSO_4 \cdot 5H_2O$, 3.8 mg/l H_3BO_3, 0.18 mg/l $(NH_4)6Mo_7 \cdot 4H_2O$). For the construction of the cDNA library, 3-week old plants were irrigated with 200 mM NaCl for 24 h before harvesting.

Directional cDNAs were synthesized (cDNA synthesis kit, Stratagene) with poly(A)+ RNA prepared from leaves of salt-treated sugar beet plants. cDNAs were ligated into phage λPG15 vector and packaged with Gigapack III gold packaging extract (Stratagene). A plasmid cDNA library was recovered from λPG15 by the *cre-lox* recombinase system (Brunelli and Pall, 1993).

Example 2: Setup of a screening assay:

The yeast strains used in this work were the wild type diploid strain W303/W303 *(can1-100,his 3-11,15,leu2-3,112, trp1-1,ura3-1,GAL+)* (WT) and a mutant deficient for glycerol phosphate dehydrogenase (*gpd1*). A diploid strain from two *gpd1* mutant strains (YRA111(W303-1A mat a *gpd1::TRP1)* and YRA114 (W303-1A *gpd1::TRP1 mat α*)) was constructed. The diploid strains were used because these prevent the isolation of recessive chromosomal mutations which might give tolerance to cold stress. The strains were grown on YPD medium (2% glucose, 2% peptone, and 1% of yeast extract) or on SD medium (2% glucose, 0.7% yeast nitrogen base (Difco) without amino acids, 50 mM MES [2-(N-morpholino)-ethanesulfonic acid] adjusted to pH 5.5 with Tris, and the required amino acids, purine and pyrimidine bases).

In a first step, the sensitivity to cold of the WT diploid strain was compared with that of the *gpd1* mutant strain. It was assumed that the production of glycerol could be a response against cold stress. Growth was monitored under cold stress conditions. Yeast strains were grown until stationary phase and 20 µl of 1/10, 1/100 and 1/1000 dilutions of the culture were spot on both YPD and on SD medium at a temperature of 15 or 10°C for 10 to 14 days, and at 30°C for 2 days as a control. 10°C was the lowest temperature measured that allowed growth of the WT strain. Surprisingly, the *gpd1* strain was shown to be cold tolerant whereas the wild type was cold sensitive (Fig. 1). This allowed the use of the WT strain for screening genes that could

confer cold tolerance, while the *gpd1* strain could serve as a standard cold tolerant yeast strain for comparative studies.

In a second step, the best conditions for transformation were determined. At the end the best protocol was: 300 ml YPD medium was inoculated with 30 µl of a saturated preculture of WT cells. The culture was grown overnight until an OD_{660}≅0.8, and centrifuged at 2000 rpm. The cells were subsequently washed with water and AcLiTE solution (0.1 M lithium acetate, 10mM Tris-HCl pH 7.6 and 1 mM EDTA (Ethylene diaminetetraacetic acid, disodium salt)). Next, the pellet of cells was resuspended in 2 ml of AcLiTE solution, incubated during 15 minutes at 30°C while shaking, whereafter 200 µl of ssDNA (10 mg/ml) was added. The cell suspension was divided in 110 µl aliquots in an Eppendorf tubes, and 200 ng of cDNA library was added. The heat shock transformation according to Gietz *et al.* (Nucleic Acids Res., **20** 1425, 1992) was used: in brief, 500 µl of PEG-AcLiTE solution (AcLiTE solution with 40% w/w of PEG (Polyethyleneglycol) 4000) was added to each aliquot. After mixing, the aliquots were incubated for 30 minutes at 30°C and next for twenty minutes at 42°C; then the cells were harvested and resuspended in 200 µl of 1M sorbitol. Two aliquots were plated in 14 cm ∅ Petri dishes with SD agar and all the necessary supplements except tryptophan (marker for the *gpd1* mutation), and uracil (marker for the plasmid). To quantify the efficiency of the transformation, four 55 µl aliquots were kept separately from the original cell pellet and were inoculated with 0, 10, 50 and 100 ng of cDNA library. Then the same transformation protocol was applied, at the end the cells were resuspended in 100 µl of sorbitol and plated in 7 cm ∅ Petri dish containing the same SD medium. The average yield was about 60 colonies per ng of cDNA. In addition it was observed that transformation with competent cells that had been frozen, or transformation in one large-scale reaction instead of many small-scale reactions dramatically decreased the yield of the transformation.

Example 3: Isolation of CRYO genes:

The cDNA library constructed in pYPGE15 was used to transform the yeast WT strain W303 by the LiCl method (Nucleic Acids Res., **20** 1425, 1992). Transformants were selected on SD plates with leucine and adenine by uracil prototrophy. Three days after transformation colonies appeared in the Petri dishes. The colonies were harvested in sterile water and the number of cells quantified by plating different dilutions. On average a 10-fold higher concentration of cells than recovered from the transformation plates was plated on YPD and SD medium. Then the plates were left in a 10°C incubator and colonies able to grow after eight days were selected. Next the tolerance of the colonies isolated in the first round was re-checked and those not giving significant tolerance were discarded. From the remaining colonies, the plasmid was

eliminated by selection in minimal medium for analysing whether the tolerance was dependent on the plasmid. As a final confirmation, the plasmid was recovered from the colonies that were able to pass the previous controls, transformed into a wild type strain and again a selection for those clones giving tolerance was performed. The results obtained are summarised in Table 1:

Table 1: Summary of the screening procedure for the selection of cold tolerant yeast transformants on YPD or SD medium.

	Number of colonies (YPD)	Number of colonies (SD)
Transformants	≅254000	≅254000
1st round isolated	68	17
Positives confirmed by retransformation	16	5

The reconfirmed positive clones were sequenced and it was shown that they encoded different genes, among those were the genes named *CRYO1*, *CRYO2*, *CRYO3*, *CRYO4* and *CRYO5* (for cryo-tolerant). Table 2:

Table 2:

Clone	Independent isolations	Highest degree of homology to:
CRYO1	8	*Arabidopsis thaliana* (*At*) protein of unknown function. *DID1* (SNF7) from yeast.
CRYO2	2	*At* protein of unknown function. Isoform of *CRYO1*.
CRYO3	4	*At* protein of unknown function. *DID2* from yeast.
CRYO4	1	Yeast *SEC17*
CRYO5	1	Ring finger domain protein

The genes encoding CRYO1 to CRYO5 proteins conferred cold stress tolerance when transferred into yeast (Fig. 2).

CRYO1 was found to be homologous to the yeast DID1 protein and, upon further analysis of the homology data, was shown to have significant homology (<90%) to the *Arabidopsis thaliana* putative proteins gi/15233464 and gi/15224854 (shown in Fig. 3). According to the invention, these putative *Arabidopsis* proteins were named AtCRYO1 and AtCRYO2 respectively.

CRYO3 is also conserved in *Arabidopsis*, with three putative proteins annotated in the database as At1g73030, At1g17730 and At4g17680, sharing more than 90% of homology. According to the invention, these putative *Arabidopsis* proteins were named AtCRYO3, AtCRYO3.2 and AtCRYO3.3 respectively. CRYO3 is also conserved in humans, and mice, as shown in the pile-up of Fig. 4.

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Example 4: Southern blotting reveals more than one isoform in sugar beet

In order to confirm the presence of *CRYO1* and *CRYO2* in the sugar beet genome and to estimate the number of genes encoding the haemoglobin in this plant species, a Southern blot analysis was performed. Genomic DNA was prepared from leaves of 3-week old sugar beet leaves (Rogers SO and Bendich AJ, Extraction of total cellular DNA from plants, algae and fungi (Eds) Plant molecular biology manual, Kluwer Academic Publishers, Dordrecht, Netherlands, 1994). 5 mg of DNA were digested with *Bam*HI, *Hind*III or *Eco*RI, electrophoresed in 0.8% agarose gel and blotted onto a nylon membrane filter (Hybond N+, Amersham Life Science). The membrane filter was hybridised with a 32P-labelled probes for *CRYO1* and *CRYO2*. Hybridisation and washes were carried out under high stringency conditions (65°C) (Church GM and Gilbert W., PNAS USA 81: 1991-1995 1984). The presence of several hybridisation fragments in all lanes, independent of the restriction endonucleases used to digest the genomic DNA, suggests that there are several isoforms in the genome, especially for *CRYO2* (Figure 5).

Example 5: CRYO2 is not induced by NaCl and ABA in sugar beet

In order to investigate whether *CRYO2* also participates in the response of sugar beet plants to salt stress, the accumulation of *CRYO2* mRNA in response to various exposure times to NaCl was analysed using northern blot analysis. Total RNA was isolated from control, 250 mM Na^+ or 100 mM ABA -treated sugar beet leaves as described by Davis et al. (Basic methods in Molecular Biology. Elsevier. Amsterdam pp.143-146 1986). 30 mg of total RNA were separated on a 1% agarose gel containing 2.2% formaldehyde and blotted onto a nylon membrane filter (Hybond N, Amersham Life Science). Hybridization using the above described probe. The *CRYO2* specific probe showed only one band that corresponded to the size of the *CRYO2* cDNA. The filter was washed twice with 4X SSC buffer (0.6 M NaCl, 0.06 M trisodium citrate adjusted to pH 7 with HCl), 0.1% SDS for 5 minutes and twice with 0.4X SSC, 0.1% SDS for five minutes at 65°C. The same filter was re-hybridized with a 1.9 kb *Eco*RI fragment comprising the α_5-tubulin gene of Arabidopsis thaliana (Ludwig *et al.*, PNAS **84** 5833 – 5837, 1987). As shown in Figure 6a the *CRYO2* mRNA did not accumulate with time upon NaCl treatment. Similarly, no induction of *CRYO2* after 3 hours of ABA treatment was observed (Figure 6b).

Example 6: CRYO5 gives also tolerance to oxidative stress.

A dilution series of W303 pYPGECRYO5 and wt yeast (control) was plated on YPD medium with 1 mM tert-butyl hydroperoxyde (t-BOOH) and tested for tolerance to oxidative stress after 2 and 4 days.

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The yeast clone with CRYO5 had a strong t-BOOH tolerance phenotype and the phenotype was very reproducible: at a concentration of 1 mM t-BOOH, control yeast cells did not grow at all, whilst yeast cells overexpressing CRYO5 did (Fig. 7).

The definition of a strong phenotype is based on drop test experiments. Different dilutions of saturated cultures (1:10, 1:100, 1:1000) were made and these were grown on selective media (YPD with 1 mM t-BOOH). "Strong phenotypes" were those clones that grew well in all the dilutions assayed. With "no strong phenotypes" is meant that the clone does not grow in all dilutions. The control cells expressing the empty plasmid did not grow at all in the selective media.

Example 7: construction of cold tolerant plants:

Plants are transformed with at least one of the CRYO genes in an expressible format under control of a constitutive or inducible promoter, using standard techniques.

Example 8: Testing of cold tolerant plants:

Transformed plants are tested by subjecting the plants to cold stress during a sufficiently long time period. When compared to the same untransformed plant line, the transformed lines show a better growth during stress conditions and/or better recovery after stress conditions and/or higher yield (biomass and/or harvestable parts).

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Claims

1. Screening method for identifying nucleic acids capable of modifying tolerance or resistance to stress conditions in plants, said method comprising the steps of:
   (i) providing a cDNA library of coding sequences from salt-treated plants;
   (ii) introducing these coding sequences in an expressible format into yeast cells;
   (iii) growing the yeast cells under conditions of stress other than salt stress;
   (iv) identifying differences between the transgenic yeast cells and wild type yeast cells, preferably identifying differences in growth rate;
   (v) isolating nucleic acids from the transgenic yeast cells that differ from the wild type yeast cells.

2. Method according to claim 1, wherein said yeast cells are *Saccharomyces cerevisiae* yeast cells, preferably *Saccharomyces cerevisiae* W303 yeast cells.

3. Method according to claim 2, wherein said salt treated plant is a halophytic plant or a part thereof, preferably *Beta vulgaris* or a part thereof.

4. Method according to claim 2 or 3, wherein the stress condition under which the yeast cells are grown is selected from temperature stress, drought stress, osmotic stress or oxidative stress, preferably said stress condition is cold stress.

5. An isolated nucleic acid selected from:
   (i) a nucleic acid encoding a protein as given in any one of SEQ ID NO 2, 4, 6, 8 or 10;
   (ii) a nucleic acid as given in any one of SEQ ID NO 1, 3, 5, 7, 9, or the complementary strand thereof;
   (iii) nucleic acids which are allelic variants to the nucleic acids defined in any of (i) to (ii);
   (iv) nucleic acids which are splice variants to the nucleic acids defined in defined in any one of (i) to (iii), and;
   (v) nucleic acids which hybridise, preferably under stringent conditions, to polynucleotides defined in (i) to (iv);
   (vi) a portion of a nucleic acid according to any of (i) to (v), which portion preferably encodes a protein having similar functional activity to the full length sequence.

6. An isolated protein selected from the group consisting of:
   (a) a polypeptide as given in any one of SEQ ID NO 2, 4, 6, 8 or 10,
   (b) a polypeptide encoded by a nucleic acid as defined in claim 5,

(c) a homologue, a derivative, an immunologically active and/or functional fragment of a protein as defined in (a) or (b).

7. A genetic construct comprising:
   (i) an isolated nucleic acid obtainable by the screening method of any of claim 1 to 4, preferably a nucleic acid encoding a protein according to claim 6;
   (ii) a regulatory element operably linked to the nucleic acid of (i), which regulatory element is preferably a plant expressible promoter; and optionally
   (iii) a transcription termination sequence.

8. A method for increasing stress tolerance of plants or yeast, comprising modulating expression in a plant or yeast of a nucleic acid as defined in claim 5 and/or modulating activity in a plant or yeast of a protein as defined in claim 6.

9. Method of claim 8, wherein said modulating expression comprises introducing into a plant or yeast a nucleic acid encoding a protein according to claim 6.

10. Method of claim 8 or 9, wherein said stress is at least one of temperature stress, osmotic stress, drought stress or oxidative stress, preferably cold stress.

11. A method for producing a transgenic plant having modified tolerance to stress relative to corresponding wild type plants, which method comprises the steps of:
   (i) introducing into a plant cell a nucleic acid encoding a protein according to claim 6; and
   (ii) cultivating said plant cell under conditions promoting regeneration and mature plant growth.

12. A plant, plant part or plant cell or yeast cell obtained by the method according to any of claims 8 to 11.

13. Plants or yeast tolerant to environmental stress, preferably cold stress, which plants or yeast have elevated levels of a protein as defined in claim 6.

14. A harvestable part, organ, tissue, propagation material, ancestors or progeny of a plant according to claim 12 or 13.

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15. A host cell comprising an isolated nucleic acid encoding a protein according to claim 6, wherein said host cell is a bacterial, yeast, fungal, plant or animal cell.

16. The use of a nucleic acid encoding a protein according to claim 6 for modifying stress tolerance in yeast, a plant, plant part or plant cell, preferably said stress is at least one of temperature stress, osmotic stress, drought stress or oxidative stress.

17. The use of a nucleic acid according to claim 5 as a selectable marker in plants or other organisms.

18. Method for increasing cold tolerance of yeast cells, comprising modulating expression in yeast of a nucleic acid encoding a glycerol phosphate dehydrogenase and/or modulating activity of a glycerol phosphate dehydrogenase.

19. Method of claim 18, wherein said expression is downregulated or inhibited.

20. Use of the *gpd1* gene for altering stress tolerance of yeast.

Abstract

Stress Tolerance

A method is presented for selecting and isolating nucleic acids capable of conferring tolerance or resistance to environmental stress conditions in plants or yeast. Furthermore, nucleic acids, the proteins they encode and their use for the production of plants or yeast with enhanced environmental stress resistance is disclosed.

088-Cryo-PROV

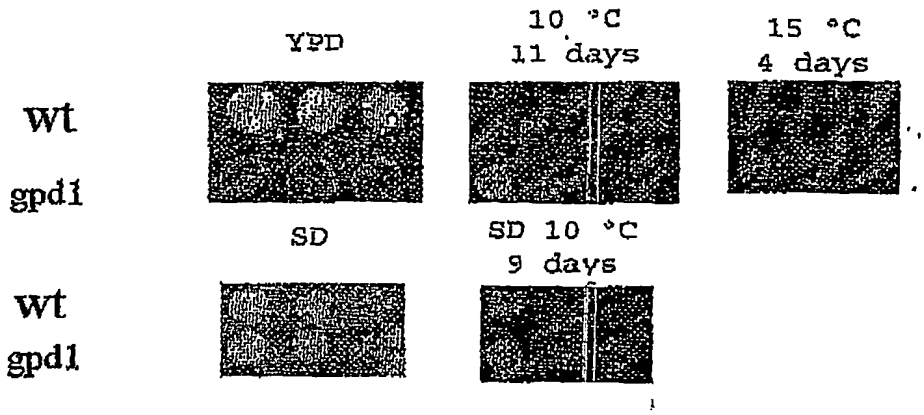

FIGURE 1

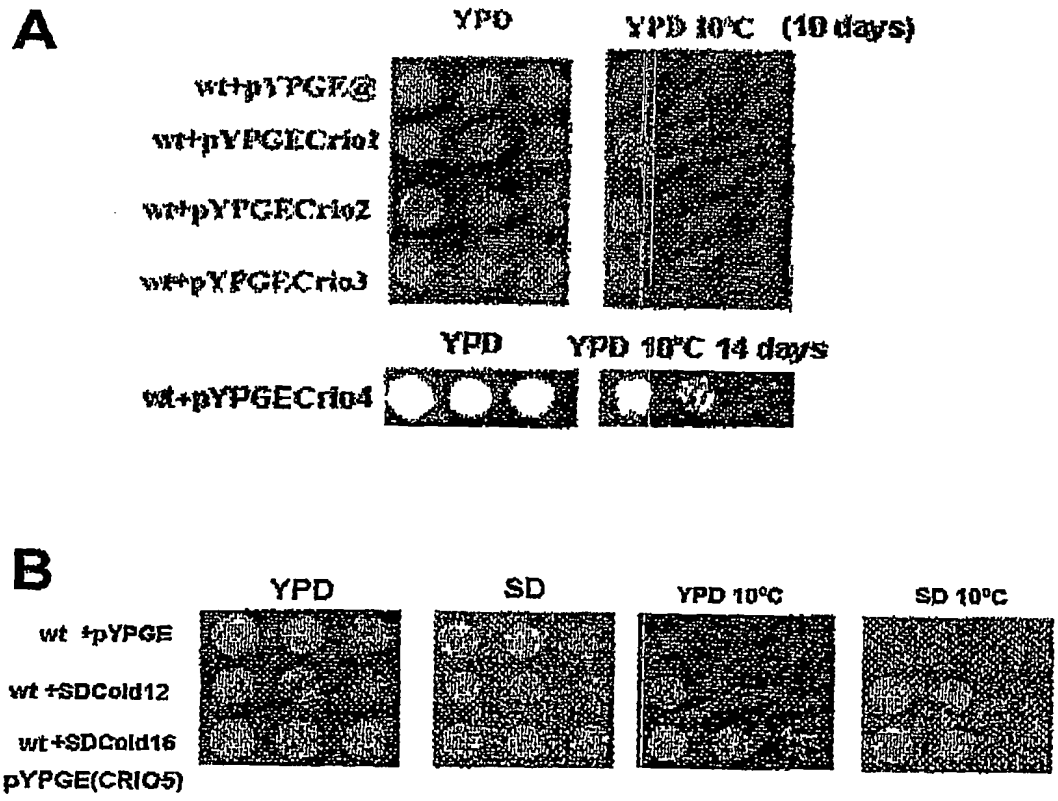

FIGURE 2

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BvCRYO2 ~MFTRVFG.K PKEGTT..SA VATLDKLSET LEMLEKKEQV LLKKAGAEVE
BvCRYO1 ~MFSRLFGAK SRDAATTETT LSTLEKLNET LEMLEKKEQL LMKKATAEVE
 scdid1 ~MWSSLFGWT SSNAKNKESP TKAIVRLREH INLLSKKQSH LRTQITNQEN

          51                                                      100
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BvCRYO2 KAKEFTRAKN KRAAITCLKR KRLYEQQIEQ LGNMQLRIHD QMILLEGAKA
BvCRYO1 KAKEFTRAKN KRAAIQCLKR KRLYEQQVEQ VGNFQLRIHD QIIMLDSAKA
 Scdid1 EARIFLTKGN KVMAKNALKK KKTIEQLLSK VEGTMESMEQ QLFSIESANL

          101                                                     150
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BvCRYO2 TTETVDALRS GASAMKAMQK A TNIDNVDK TMDEIENQTE NLKQIQEALSAPIGAAD
BVCRYO1 TTETVAALRS GASAMKAMQK A.TNIDDVDK TMDEINEQTD NLRQIRRH
 Scdid1 NLETMRAMQE GAKAMKTIH. SGLDIDKVDE TMDEIREQVE LGDEISDAIS
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FIGURE 3

```
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 AtCRYO3 ~~MGNTDKLM NQIFDLKFTS KSLQRQSRKC EKEEKAEKLK VKKAIEKGNM
 BvCRYO3 ~~MGNTEKLM NQIMELKFTS KSLQRQSRKC EKEEKAEKLK VKKAIEKGNM
 DeCRYO3 ~~~~~~~~ME NQLFQLKFTS KQLEKQSKKS EQSEKAQKIK LKKAIEQGNM
 MmCRYO3 ~~~~~~~~MD DTLFQLKFTA KQLEKLAKKA EKDSKAEQAK VKKALQQKNV
 HsCRYO3 ~~~~~~~~MD DTLFQLKFTA KQLEKLAKKA EKDSKAEQAK VKKALLQKNV
 ScCRYO3 MSRNSAAGLE NTLFQLKFTS KQLQKQANKA SKEEKQETNK LKRAL.NENE
 SpCRYO3 ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~MSL SMNFFTAHLS IAIAITKGNS

          51                                                         100
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 AtCRYO3 DGARIYAENA IRKRSEQMNY LRLASRLDAV VARLDTQAKM TTITKSMTNI
 BvCRYO3 DGARIYAENA IRKRTEQMNY LRLASRLDAV VSRLDTQAKM QTIGKSMGSI
 DeCRYO3 DGARIYAQNA IREKNQSLNY LRLASRIDAV ASRVETAIRM KSVTGSMANI
 MmCRYO3 ECARVYAENA IRKKNEGVNW LRMASRVDAV ASKVQTAVTM KGVTKNMAQV
 HsCRYO3 ECARVYAENA IRKKNEGVNW LRMASRVDAV ASKVQTAVTM KGVTKNMAQV
 ScCRYO3 DISRIYASNA IRKKNERLQL LKLASRVDSV ASRVQTAVTM RQVSASMGQV
 SpCRYO3 EIARIYASNA IRKQQESLNL LKLSSRIDAV SSRLQTAVTM RAVSGNMAGV

          101                                                        150
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 AtCRYO3 VKSLESSLAT GNLQKMSETM DSFEKQFVNM EVQAEFMENA MAGSTSLSTP
 BvCRYO3 VKSLESSLNT GNLQKMSETM DNFEKQFVNM EVQAEFMESS MAGSTSLSTP
  DeDID2 VKSMEKSMRN MDLEKITQVM DQFERQFEDL DVQSVYVENA MNQTTTLSTP
  MmDID2 TKALDKALSA MDLQKVSAVM DRFEQQVQNL DVHTSVMEDS VSSATTLTTP
  HsDID2 TKALDKALST MDLQKVSSVM DRFEQQVQNI DVHTSVMEDS MSSATTLTTP
  ScDID2 CKGMDKALQN MNLQQITMIM DKFEQQFEDL DTSVNVYEDM GVNSDAMLVD
  SpDID2 VRGMDRAMKT MNLEMISQVM DKFEAQFDDV NVQTGYMNKA MGSVTAVDTP

          151                                                        200
AtCRYO3.2EGEVNSLMQQ VADDYGLEV. .SVGLPQ.PA GHAIPTKTEE KVEEDDLTRR
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 AtCRYO3 EGEVNSLMQQ VADDYGLEV. .SVGLPQ.PA GHAIPTKTEE KVDEDDLSRR
 BvCRYO3 ETEVNSLMQQ VADDYGLEG. .SVGLPQ.AA GHAIPV...P KAAEKVDEG*
  DeDID2 ADQVDLLISQ VADEHGL... .NVGMQM... GSA.PSEKVQ QGETDELTER
  MmDID2 QEQVDSLIVQ IAEENGLEVL D..QLSQLPE GASAVGESSV RSQEDQLSRR
  HsDID2 QEQVDSLIMQ IAEENGLEVL D..QLSQLPE GASAVGESSV RSQEDQLSRR
  ScDID2 NDKVDELMSK VADENGMELK QSAKLDNVPE .IKAKEVNVD DEKEDKLAQR
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FIGURE 4

088-Cryo-PROV

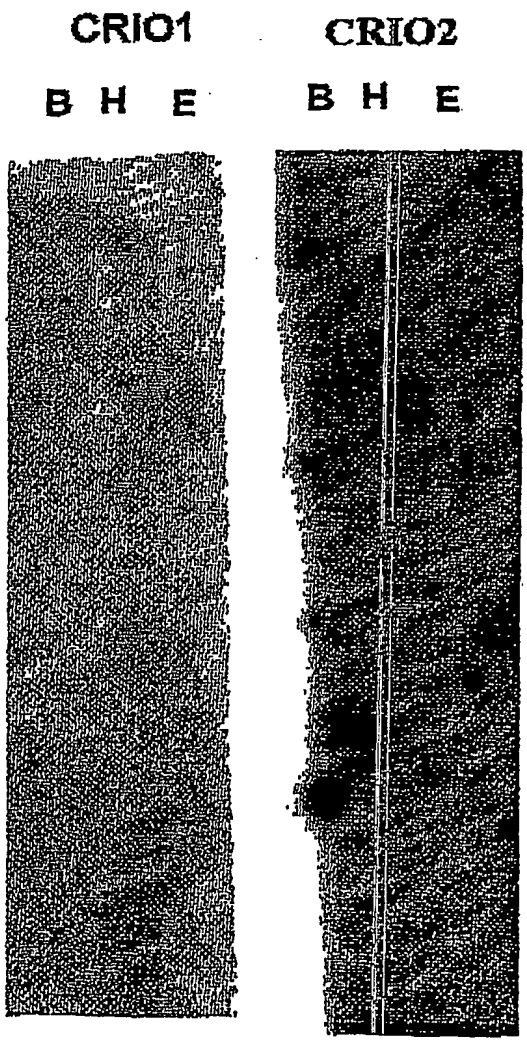
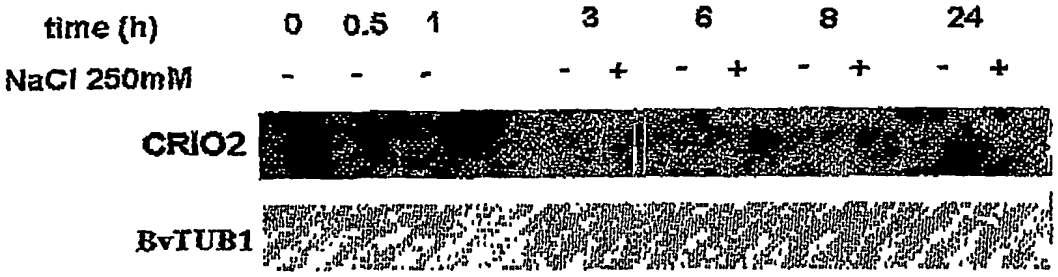


FIGURE 5

088-Cryo-PROV



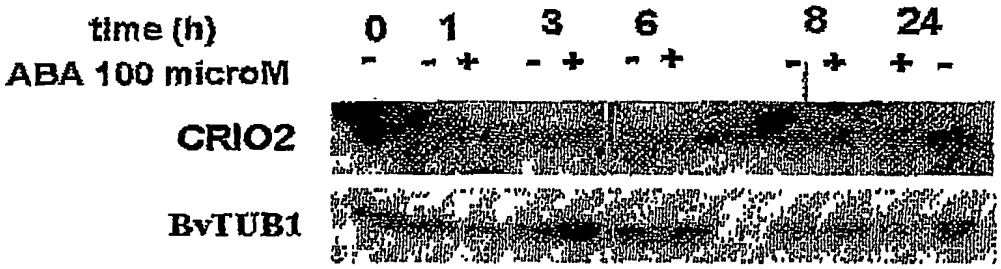


FIGURE 6

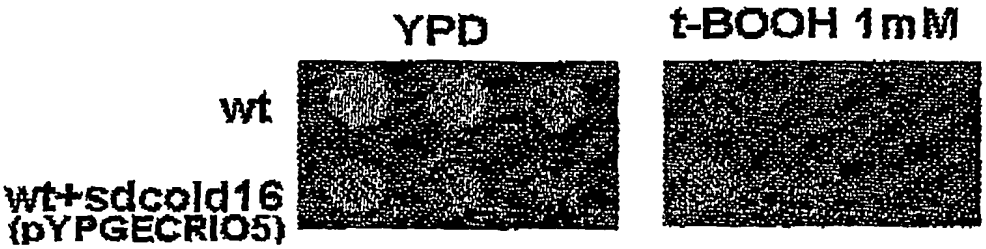


FIGURE 7

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acgatcgtag agtctctgtt tttcactgta taaatctatt caaacaattt tclctctcct 180

attatttcaa tttcggtttg ctaaltcaag gtgaatcaa atg tcg gca aat atg 234
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1 5

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act act tta tct aca tta gag aaa ttg aat gag aca ctt gaa atg cta       330
Thr Thr Leu Ser Thr Leu Glu Lys Leu Asn Glu Thr Leu Glu Met Leu
             25                  30                  35

gag aag aaa gag cag ctt cta atg aaa aag gct act gca gag gtt gaa       378
Glu Lys Lys Glu Gln Leu Leu Met Lys Lys Ala Thr Ala Glu Val Glu
         40                  45                  50

aag gcc aaa gag ttc aca agg gca aag aat aaa cgt gct gct ata caa       426
Lys Ala Lys Glu Phe Thr Arg Ala Lys Asn Lys Arg Ala Ala Ile Gln
     55                  60                  65

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Cys Leu Lys Arg Lys Arg Leu Tyr Glu Gln Gln Val Glu Gln Val Gly
 70                  75                  80                  85

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Leu Gln Pro Phe Thr Thr Ala Pro Thr Ala Pro Ile His Val Pro Glu
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Thr Ala Glu Val Glu Lys Ala Lys Glu Phe Thr Arg Ala Lys Asn Lys
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Val Glu Gln Val Gly Asn Phe Gln Leu Arg Ile His Asp Gln Ile Ile
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Met Leu Asp Ser Ala Lys Ala Thr Thr Glu Thr Val Ala Ala Leu Arg
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115 120 125

Asp Val Asp Lys Thr Met Asp Glu Ile Asn Glu Gln Thr Asp Asn Leu
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Arg Gln Ile Gln Glu Ala Leu Ala Thr Pro Val Gly Ala Thr Asp Phe
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Asp Glu Asp Glu Leu Glu Ala Glu Leu Glu Glu Leu Glu Gly Ala Glu
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Leu Glu Glu Gln Leu Leu Gln Pro Phe Thr Thr Ala Pro Thr Ala Pro
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gga aac atg cag ttg cga att cat gat cag atg ata ctg ctt gaa ggg      591
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Gln Leu Arg Ile His Asp Gln Met Ile Leu Leu Glu Gly Ala Lys Ala
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          5                  10                  15

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Ser Lys Ser Leu Gln Arg Gln Ser Arg Lys Cys Glu Lys Glu Glu Lys
      20                  25                  30

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Ala Glu Lys Leu Lys Val Lys Lys Ala Ile Glu Lys Gly Asn Met Asp
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ccctttttcct ttgtcgatat cntgtaatta gttaggggggt                       1019
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<223> unknown nucleotide

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Met Gly Asn Thr Glu Lys Leu Met Asn Gln Ile Met Glu Leu Lys Phe
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Thr Ser Lys Ser Leu Gln Arg Gln Ser Arg Lys Cys Glu Lys Glu Glu
20 25 30

Lys Ala Glu Lys Leu Lys Val Lys Lys Ala Ile Glu Lys Gly Asn Met
35 40 45

Asp Gly Ala Arg Ile Tyr Ala Glu Asn Ala Ile Arg Lys Arg Thr Glu
50 55 60

Gln Met Asn Tyr Leu Arg Leu Ala Ser Arg Leu Asp Ala Val Val Ser
65 70 75 80

Arg Leu Asp Thr Gln Ala Lys Met Gln Thr Ile Gly Lys Ser Met Gly
85 90 95

Ser Ile Val Lys Ser Leu Glu Ser Ser Leu Asn Thr Gly Asn Leu Gln
100 105 110

Lys Met Ser Glu Thr Met Asp Asn Phe Glu Lys Gln Phe Val Asn Met
115 120 125

Glu Val Gln Ala Glu Phe Met Glu Ser Ser Met Ala Gly Ser Thr Ser
130 135 140

Leu Ser Thr Pro Glu Thr Glu Val Asn Ser Leu Met Gln Gln Val Ala
145 150 155 160

Asp Asp Tyr Gly Leu Glu Val Ser Val Gly Leu Pro Gln Ala Ala Gly
165 170 175

His Ala Ile Pro Val Pro Lys Ala Ala Glu Lys Val Asp Glu Asp Asp
180 185 190

Leu Thr Arg Arg Leu Ala Glu Leu Lys Ala Arg Gly
195 200

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088-Cryo-PROV2.ST25.txt

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agatcaccc atg gcg gaa gaa acc cat aag cca gaa tca acg gtg gct gaa     111
          Met Ala Glu Glu Thr His Lys Pro Glu Ser Thr Val Ala Glu
          1               5                  10

gtg gtg gtt cca gta gcc gag aaa cca gct gag aag cca gct gag aag      159
Val Val Val Pro Val Ala Glu Lys Pro Ala Glu Lys Pro Ala Glu Lys
15                  20                  25                  30

gca gtt ctc cca cct gaa gct gag aaa cta gct gca gct gaa tca gct      207
Ala Val Leu Pro Pro Glu Ala Glu Lys Leu Ala Ala Ala Glu Ser Ala
                35                  40                  45

gaa gcc gag aag cca gct gat tca gcc gag gct aag ata gct caa caa      255
Glu Ala Glu Lys Pro Ala Asp Ser Ala Glu Ala Lys Ile Ala Gln Gln
            50                  55                  60

gtc tca ttc aaa gag gag act aat gtt gca agt gag cta cct gag cta      303
Val Ser Phe Lys Glu Glu Thr Asn Val Ala Ser Glu Leu Pro Glu Leu
        65                  70                  75

cat aga aag gct ctc gag gac ttg aag aaa ctt att caa gaa gcc ctc      351
His Arg Lys Ala Leu Glu Asp Leu Lys Lys Leu Ile Gln Glu Ala Leu
    80                  85                  90

gag aag cac gag ttc tct tct cct cct cct ccg cct ccg cct gct cca      399
Glu Lys His Glu Phe Ser Ser Pro Pro Pro Pro Pro Pro Pro Ala Pro
95                  100                 105                 110

gct aaa gtt gag gag aag gcg gaa gag aag aaa gag gaa caa cct cca      447
Ala Lys Val Glu Glu Lys Ala Glu Glu Lys Lys Glu Glu Gln Pro Pro
                115                 120                 125

tcc acc acc tcc acc acc acc acc acc acc gcg gtt tca gat gag      495
Ser Thr Thr Ser Thr Thr Thr Thr Thr Thr Ala Val Ser Asp Glu
            130                 135                 140

gtt gct gtt gct cct cca tcc gaa gag gcc ccg aaa act gac gag gcc      543
Val Ala Val Ala Pro Pro Ser Glu Glu Ala Pro Lys Thr Asp Glu Ala
        145                 150                 155

tct ccg aaa gtg cag gag gag cct gca aaa ata gtt gag caa cca cct      591
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Ser Pro Lys Val Glu Glu Glu Pro Ala Lys Ile Val Glu Gln Pro Pro
160                 165                 170

aca acc ccg gca gaa gaa cct gaa cca gca aaa aca cct gag gtt gtt      639
Thr Thr Pro Ala Glu Glu Pro Glu Pro Ala Lys Thr Pro Glu Val Val
175                 180                 185                 190

gtt gct gaa gag gag aaa act ggt gag gat att aaa gaa act ata gta      687
Val Ala Glu Glu Glu Lys Thr Gly Glu Asp Ile Lys Glu Thr Ile Val
                195                 200                 205

gtc gag gtt gcg aca act acc gca gca cca gta cta acc gaa cca gaa      735
Val Glu Val Ala Thr Thr Thr Ala Ala Pro Val Leu Thr Glu Pro Glu
            210                 215                 220

tct gtt gag gag acc cca aaa gaa gct gaa gtt gta gtg gaa gaa tca      783
Ser Val Glu Glu Thr Pro Lys Glu Ala Glu Val Val Val Glu Glu Ser
        225                 230                 235

cca aag gag cca gaa gaa gta tca ata tgg ggc att cca ctt ctt gct      831
Pro Lys Glu Pro Glu Glu Val Ser Ile Trp Gly Ile Pro Leu Leu Ala
    240                 245                 250

gat gaa aga agt gat gta att cta ttg aaa ttc tta aga gca aga gat      879
Asp Glu Arg Ser Asp Val Ile Leu Leu Lys Phe Leu Arg Ala Arg Asp
255                 260                 265                 270

tat aga gtg aaa gat gct ttc act atg att agc aat act gct cgt tgg      927
Tyr Arg Val Lys Asp Ala Phe Thr Met Ile Arg Asn Thr Ala Arg Trp
                275                 280                 285

aga aaa gaa ttt gag gtt gat tct tta ctt gat gaa gat ctt gga aat      975
Arg Lys Glu Phe Glu Val Asp Ser Leu Leu Asp Glu Asp Leu Gly Asn
            290                 295                 300

gat tat gag aaa gtt gtt ttt aca cat gga gtt gat aaa caa ggt cgt     1023
Asp Tyr Glu Lys Val Val Phe Thr His Gly Val Asp Lys Gln Gly Arg
        305                 310                 315

cct gtt tgt tat aat gtg ttt gga gag ttt caa aat aag gaa ctt tat     1071
Pro Val Cys Tyr Asn Val Phe Gly Glu Phe Gln Asn Lys Glu Leu Tyr
    320                 325                 330

cag aat act ttc tct gat gca gaa aaa agg aaa aag ttc ttg aga tgg     1119
Gln Asn Thr Phe Ser Asp Ala Glu Lys Arg Lys Lys Phe Leu Arg Trp
335                 340                 345                 350

ttg att caa ttc ctt gaa aaa act att aga act ctt gat ttt agt cct     1167
Leu Ile Gln Phe Leu Glu Lys Thr Ile Arg Thr Leu Asp Phe Ser Pro
                355                 360                 365

gaa ggc att aat tct ttt gtt ctt gtt aat gat ttg aag aat tct cct     1215
Glu Gly Ile Asn Ser Phe Val Leu Val Asn Asp Leu Lys Asn Ser Pro
            370                 375                 380

ggg tat ggt aag aga gat ctt tac aaa gtt att gac aag ttt ctt gag     1263
Gly Tyr Gly Lys Arg Asp Leu Tyr Lys Val Ile Asp Lys Phe Leu Glu
        385                 390                 395

att ctc cag gat aat tac cca gaa ttt gct gct aaa cag ttg tgc atc     1311
Ile Leu Gln Asp Asn Tyr Pro Glu Phe Ala Ala Lys Gln Leu Cys Ile
    400                 405                 410

aat gtt tca tgg tgg tct tgg cat aca act gga tct att tga             1353
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Asn Val Ser Trp Trp Ser Trp His Thr Thr Gly Ser Ile
415 420 425

ctgtattiac accaaggagc aagagcaagt ttgtgtttgc aagcccatct aaaactgctg 1413

agacccttttt caagtacata gctcctgagc aggtgcctgt tcaatttggt gggcacagca 1473

agtttggcga gcatgagttt tcccctgctg atactgt 1510

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<222> (2)..(3)

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Val Pro Val Ala Glu Lys Pro Ala Glu Lys Pro Ala Glu Lys Ala Val
20 25 30

Leu Pro Pro Glu Ala Glu Lys Leu Ala Ala Ala Glu Ser Ala Glu Ala
35 40 45

Glu Lys Pro Ala Asp Ser Ala Glu Ala Lys Ile Ala Gln Gln Val Ser
50 55 60

Phe Lys Glu Glu Thr Asn Val Ala Ser Glu Leu Pro Glu Leu His Arg
65 70 75 80

Lys Ala Leu Glu Asp Leu Lys Lys Leu Ile Gln Glu Ala Leu Glu Lys
85 90 95

His Glu Phe Ser Ser Pro Pro Pro Pro Pro Pro Pro Ala Pro Ala Lys
100 105 110

Val Glu Glu Lys Ala Glu Glu Lys Lys Glu Glu Gln Pro Pro Ser Thr
115 120 125

Thr Ser Thr Thr Thr Thr Thr Thr Thr Ala Val Ser Asp Glu Val Ala
130 135 140

Val Ala Pro Pro Ser Glu Glu Ala Pro Lys Thr Asp Glu Ala Ser Pro
145 150 155 160

Lys Val Glu Glu Glu Pro Ala Lys Ile Val Glu Gln Pro Pro Thr Thr
165 170 175

Pro Ala Glu Glu Pro Glu Pro Ala Lys Thr Pro Glu Val Val Val Ala
180 185 190

Glu Glu Glu Lys Thr Gly Glu Asp Ile Lys Glu Thr Ile Val Val Glu
195 200 205

Val Ala Thr Thr Thr Ala Ala Pro Val Leu Thr Glu Pro Glu Ser Val
210 215 220

Glu Glu Thr Pro Lys Glu Ala Glu Val Val Val Glu Glu Ser Pro Lys
225 230 235 240

Glu Pro Glu Glu Val Ser Ile Trp Gly Ile Pro Leu Leu Ala Asp Glu
245 250 255

Arg Ser Asp Val Ile Leu Leu Lys Phe Leu Arg Ala Arg Asp Tyr Arg
260 265 270

Val Lys Asp Ala Phe Thr Met Ile Arg Asn Thr Ala Arg Trp Arg Lys
275 280 285

Glu Phe Glu Val Asp Ser Leu Leu Asp Glu Asp Leu Gly Asn Asp Tyr
290 295 300

Glu Lys Val Val Phe Thr His Gly Val Asp Lys Gln Gly Arg Pro Val
305 310 315 320

Cys Tyr Asn Val Phe Gly Glu Phe Gln Asn Lys Glu Leu Tyr Gln Asn
325 330 335

Thr Phe Ser Asp Ala Glu Lys Arg Lys Lys Phe Leu Arg Trp Leu Ile
340 345 350

Gln Phe Leu Glu Lys Thr Ile Arg Thr Leu Asp Phe Ser Pro Glu Gly
355 360 365

Ile Asn Ser Phe Val Leu Val Asn Asp Leu Lys Asn Ser Pro Gly Tyr
370 375 380

Gly Lys Arg Asp Leu Tyr Lys Val Ile Asp Lys Phe Leu Glu Ile Leu
385 390 395 400

Gln Asp Asn Tyr Pro Glu Phe Ala Ala Lys Gln Leu Cys Ile Asn Val
405 410 415

Ser Trp Trp Ser Trp His Thr Thr Gly Ser Ile
420 425

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<211> 2052

<212> DNA

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ggaaggccgc ctctttttg ggtcgctgac tattaactga aactttgtaa atctactc 118

atg gat gaa tat tcc aat aga aaa tct tct ggt ctt gct atc tcc agg 166
Met Asp Glu Tyr Ser Asn Arg Lys Ser Ser Gly Leu Ala Ile Ser Arg
1 5 10 15

aga ggg cct agc ctt gtt tta agg gac tca gcg gag aac aac aaa gat 214
Arg Gly Pro Ser Leu Val Leu Arg Asp Ser Ala Glu Asn Asn Lys Asp
20 25 30

cgg aat gtt cag gtt tgc agc cga gtt gga tgt ggc agc aag ttg aat 262
Arg Asn Val Gln Val Cys Ser Arg Val Gly Cys Gly Ser Lys Leu Asn
35 40 45

tca gtg aag gat gct aaa gtt agc tct ccg agt aaa gtc aaa tct cca 310
Ser Val Lys Asp Ala Lys Val Ser Ser Pro Ser Lys Val Lys Ser Pro
50 55 60

aaa act cct ttc cgt tca tct gct caa gga aaa gaa acc att gga agt 358
Lys Thr Pro Phe Arg Ser Ser Ala Gln Gly Lys Glu Thr Ile Gly Ser
65 70 75 80

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tca tcc aga act ctg gct tct cct agt cct ttt aaa aaa tct ctt tca      406
Ser Ser Arg Thr Leu Ala Ser Pro Ser Pro Phe Lys Lys Ser Leu Ser
                85                  90                  95

gac cgg aag aaa aaa ctg cct ctt aat ctt gac act gat tca gaa atg      454
Asp Arg Lys Lys Lys Leu Pro Ser Asn Leu Asp Thr Asp Ser Glu Met
            100                 105                 110

tgc agt ctt caa gat gaa tcc gag gaa gtc tct gga aag acc cgg ata      502
Cys Ser Leu Gln Asp Glu Ser Glu Glu Val Ser Gly Lys Thr Arg Ile
        115                 120                 125

agg gtt cag ccc gag cca gaa gat cat gat tcc att gaa gct tca tca      550
Arg Val Gln Pro Glu Pro Glu Asp His Asp Ser Ile Glu Ala Ser Ser
    130                 135                 140

tct gaa gct ggg agt tcc agt tcg gga ccc tct aac aga ttg gca aac      598
Ser Glu Ala Gly Ser Ser Ser Ser Gly Pro Ser Asn Arg Leu Ala Asn
145                 150                 155                 160

aga aat act cag agg ttt ggg ttg ggg cgc caa gat tct gct gca agt      646
Arg Asn Thr Gln Arg Phe Gly Leu Gly Arg Gln Asp Ser Ala Ala Ser
                165                 170                 175

tct gct tca ttt tct tta aat aaa acc aac caa ggg caa aga aat ggt      694
Ser Ala Ser Phe Ser Leu Asn Lys Thr Asn Gln Gly Gln Arg Asn Gly
            180                 185                 190

ggt ggt ggt ggt gct agt gct aac agg tat aat ctg cga caa tta aaa      742
Gly Gly Gly Gly Ala Ser Ala Asn Arg Tyr Asn Leu Arg Gln Leu Lys
        195                 200                 205

tgt aac tca atc tct gac gtt gtt cca tca ggt tct ccg cag tct gct      790
Cys Asn Ser Ile Ser Asp Val Val Pro Ser Gly Ser Pro Gln Ser Ala
    210                 215                 220

gaa tca agt ctc agt aag aag agg gac aca ggt tgt agg aag aga aat      838
Glu Ser Ser Leu Ser Lys Lys Arg Asp Thr Gly Cys Arg Lys Arg Asn
225                 230                 235                 240

ggt gaa gct gag agt agt tta cct gtg aga ggt aag aaa att aat ggg      886
Gly Glu Ala Glu Ser Ser Leu Pro Val Arg Gly Lys Lys Ile Asn Gly
                245                 250                 255

gca acc caa gat gat agg agg aat gac tat cca aat cgt gga ata tca      934
Ala Thr Gln Asp Asp Arg Arg Asn Asp Tyr Pro Asn Arg Gly Ile Ser
            260                 265                 270

ata tct gat aca agg cgt acc aga agc tcg agt cct ggg aat aac gat      982
Ile Ser Asp Thr Arg Arg Thr Arg Ser Ser Ser Pro Gly Asn Asn Asp
        275                 280                 285

gtc acg tct gtt agg agt cgg aga tct gtt gct aga aca agg ctt tca     1030
Val Thr Ser Val Arg Ser Arg Arg Ser Val Ala Arg Thr Arg Leu Ser
    290                 295                 300

aat cag gat acc cgg gat aga tta cca ttg gtt gag tca ccc ctg agg     1078
Asn Gln Asp Thr Arg Asp Arg Leu Pro Leu Val Glu Ser Pro Leu Arg
305                 310                 315                 320

aac cca tct tca cct cta ccc gag tca tca act gga gga act gat ttt     1126
Asn Pro Ser Ser Pro Leu Pro Glu Ser Ser Thr Gly Gly Thr Asp Phe
                325                 330                 335
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agt ttg gaa aat cag ttc tct ggc cga act cca gct cgt tct tta agt      1174
Ser Leu Glu Asn Gln Phe Ser Gly Arg Thr Pro Ala Gly Ser Leu Ser
            340                 345                 350

tct tat aat aga cca ggt ggc ggt agt gaa cat atg cgg cct agt agg      1222
Ser Tyr Asn Arg Pro Gly Gly Gly Ser Glu His Met Arg Pro Ser Arg
        355                 360                 365

tct att gat ccc tat gaa gct ggc att gct cgc tct ttt atg aac cgt      1270
Ser Ile Asp Pro Tyr Glu Ala Gly Ile Ala Arg Ser Phe Met Asn Arg
    370                 375                 380

gat acc tta aga cag tac aac tta gat ggg att gca gag atg tta tta      1318
Asp Thr Leu Arg Gln Tyr Asn Leu Asp Gly Ile Ala Glu Met Leu Leu
385                 390                 395                 400

gct cta gag aga att gaa caa gaa gaa gat cca acc tat gag caa ttg      1366
Ala Leu Glu Arg Ile Glu Gln Glu Glu Asp Pro Thr Tyr Glu Gln Leu
                405                 410                 415

ctt gtt ctg gag act aat ctt ttc cta gga gga ctt tct ttt cat gat      1414
Leu Val Leu Glu Thr Asn Leu Phe Leu Gly Gly Leu Ser Phe His Asp
            420                 425                 430

cag cac agg gac atg agg ctg gat att gat aat atg tca tat gag gaa      1462
Gln His Arg Asp Met Arg Leu Asp Ile Asp Asn Met Ser Tyr Glu Glu
        435                 440                 445

cta tta gct tta gaa gag agc atg gga act gta aga cag ccg tgc cag      1510
Leu Leu Ala Leu Glu Glu Ser Met Gly Thr Val Arg Gln Pro Cys Gln
    450                 455                 460

aag atg att tgg cta agt gtc tta aaa gga aca tct acc agg gtg ttg      1558
Lys Met Ile Trp Leu Ser Val Leu Lys Gly Thr Ser Thr Arg Val Leu
465                 470                 475                 480

cag att gta gag agg atg agc atg ata tca aat gca gca tat gcc agg      1606
Gln Ile Val Glu Arg Met Ser Met Ile Ser Asn Ala Ala Tyr Ala Arg
                485                 490                 495

aag aat atg gtg gcg ggg aag aag tag gaagattgag ttgtgatcac            1653
Lys Asn Met Val Ala Gly Lys Lys
            500

agctaccaca ttgaatgtat aaatcaatgg ttgaggctca agaactggtg ccctatctgc    1713

aaggcttctg catcaccttc aacttcagca actccgcctc cctgaacttc gctgttatat    1773

tcttcccttt tttttccagt ttgtacagac cggaatctgt cgatttttat ttcttcatca    1833

gaaatttgat gtttctatag atagtccttt ggttactatt ttctttttcc ttatttgtac    1893

atataatttc tcttctatgt gccaactaat aatgctcgag ctgttagaag ctccagtatg    1953

ggaacaggtt cacttcactt attttacata aacagattct caagtatata taaatcccta    2013

tcctcaaaaa aaaaaaaaaa aaacccgagg gggggngcg                           2052
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Met Asp Glu Tyr Ser Asn Arg Lys Ser Ser Gly Leu Ala Ile Ser Arg
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Arg Gly Pro Ser Leu Val Leu Arg Asp Ser Ala Glu Asn Asn Lys Asp
20 25 30

Arg Asn Val Gln Val Cys Ser Arg Val Gly Cys Gly Ser Lys Leu Asn
35 40 45

Ser Val Lys Asp Ala Lys Val Ser Ser Pro Ser Lys Val Lys Ser Pro
50 55 60

Lys Thr Pro Phe Arg Ser Ser Ala Gln Gly Lys Glu Thr Ile Gly Ser
65 70 75 80

Ser Ser Arg Thr Leu Ala Ser Pro Ser Pro Phe Lys Lys Ser Leu Ser
85 90 95

Asp Arg Lys Lys Lys Leu Pro Ser Asn Leu Asp Thr Asp Ser Glu Met
100 105 110

Cys Ser Leu Gln Asp Glu Ser Glu Glu Val Ser Gly Lys Thr Arg Ile
115 120 125

Arg Val Gln Pro Glu Pro Glu Asp His Asp Ser Ile Glu Ala Ser Ser
130 135 140

Ser Glu Ala Gly Ser Ser Ser Ser Gly Pro Ser Asn Arg Leu Ala Asn
145 150 155 160

Arg Asn Thr Gln Arg Phe Gly Leu Gly Arg Gln Asp Ser Ala Ala Ser
165 170 175

Ser Ala Ser Phe Ser Leu Asn Lys Thr Asn Gln Gly Gln Arg Asn Gly
180 185 190

Gly Gly Gly Gly Ala Ser Ala Asn Arg Tyr Asn Leu Arg Gln Leu Lys
195 200 205

Cys Asn Ser Ile Ser Asp Val Val Pro Ser Gly Ser Pro Gln Ser Ala
210 215 220

Glu Ser Ser Leu Ser Lys Lys Arg Asp Thr Gly Cys Arg Lys Arg Asn
225 230 235 240

Gly Glu Ala Glu Ser Ser Leu Pro Val Arg Gly Lys Lys Ile Asn Gly
245 250 255

Ala Thr Gln Asp Asp Arg Arg Asn Asp Tyr Pro Asn Arg Gly Ile Ser
260 265 270

Ile Ser Asp Thr Arg Arg Thr Arg Ser Ser Ser Pro Gly Asn Asn Asp
275 280 285

Val Thr Ser Val Arg Ser Arg Arg Ser Val Ala Arg Thr Arg Leu Ser
290 295 300

Asn Gln Asp Thr Arg Asp Arg Leu Pro Leu Val Glu Ser Pro Leu Arg
305 310 315 320

Asn Pro Ser Ser Pro Leu Pro Glu Ser Ser Thr Gly Gly Thr Asp Phe
325 330 335

Ser Leu Glu Asn Gln Phe Ser Gly Arg Thr Pro Ala Gly Ser Leu Ser
340 345 350

Ser Tyr Asn Arg Pro Gly Gly Gly Ser Glu His Met Arg Pro Ser Arg
355 360 365

Ser Ile Asp Pro Tyr Glu Ala Gly Ile Ala Arg Ser Phe Met Asn Arg
370 375 380

Asp Thr Leu Arg Gln Tyr Asn Leu Asp Gly Ile Ala Glu Met Leu Leu
385 390 395 400

Ala Leu Glu Arg Ile Glu Gln Glu Glu Asp Pro Thr Tyr Glu Gln Leu
405 410 415

Leu Val Leu Glu Thr Asn Leu Phe Leu Gly Gly Leu Ser Phe His Asp
420 425 430

Gln His Arg Asp Met Arg Leu Asp Ile Asp Asn Met Ser Tyr Glu Glu
435 440 445

Leu Leu Ala Leu Glu Glu Ser Met Gly Thr Val Arg Gln Pro Cys Gln
450 455 460

Lys Met Ile Trp Leu Ser Val Leu Lys Gly Thr Ser Thr Arg Val Leu
465 470 475 480

Gln Ile Val Glu Arg Met Ser Met Ile Ser Asn Ala Ala Tyr Ala Arg
485 490 495

Lys Asn Met Val Ala Gly Lys Lys
500


EP2004050513

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